PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12Q 1/68, C12P 19/34, C12N 15/11, 15/85, C07H 21/04

A1

(11) International Publication Number: WO 99/18238

(43) International Publication Date: 15 April 1999 (15.04.99)

(21) International Application Number: PCT/US98/20361

(22) International Filing Date: 28 September 1998 (28.09.98)

(30) Priority Data:

60/060,673	2 October 1997 (02.10.97)	US
09/136,080	18 August 1998 (18.08.98)	US

(63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Applications

US	60/060,673 (CIP)
Filed on	2 October 1997 (02.10.97)
US	09/136,080 (CIP)
Filed on	18 August 1998 (18.08.98)

(71) Applicant *(for all designated States except US)*: OASIS BIOSCIENCES, INC. [US/US]; 445 W. Willowspring Drive, Encinitas, CA 92024–1290 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: RILEY, Timothy, A. [US/US]; 12894 War Horse Street, San Diego, CA 92129 (US). BROWN, Bob, D. [US/US]; 445 North Willowspring Drive, Encinitas, CA 92024–1920 (US). ARNOLD, Lyle, J. [US/US]; 15638 Boulder Moutain Road, Poway, CA 92064 (US).

(74) Agent: WETHERELL, John, R., Jr.; Fish & Richardson P.C., Suite 1400, 4225 Executive Square, La Jolla, CA 92037 (US).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: COMBINATORIAL ANTISENSE LIBRARY

(57) Abstract

Combinatorial libraries comprise first oligonucleotide analogs and second oligonucleotide analogs which are coupled together to form antisense molecules capable of binding target polynucleotides and activating an RNase, and ribozymes capable of cleaving polynucleotides.

COMBINATORIAL ANTISENSE LIBRARY

Field of the Invention

This invention relates generally to the fields of organic chemistry and biological assays. More specifically, the invention relates to methods and compositions for determining optimal antisense sequences, and optimized libraries of oligonucleotide analogs.

Background of the Invention

Antisense technology is based on the finding that DNA and/or RNA transcription or translation can be modulated using an oligonucleotide which binds to the target nucleic acid. By exploiting the Watson-Crick base pairing, one can design antisense molecules having a very high degree of specificity for the target nucleic acid. Oligonucleotides having only standard ("natural") bases and backbones must in general contain at least 17 bases in order to bind with sufficient energy to effectively down-regulate gene expression by activating RNase H.

However, even given DNA or RNA of known sequence, it is still difficult to design an optimally effective antisense molecule. This is because nucleic acids are subject to the formation of a variety of secondary and tertiary structures in vivo, and are frequently coiled, supercoiled, folded, and/or obscured by proteins. Some portions of the target sequence are much more susceptible to binding and hybridization by antisense molecules, while other portions of the target sequence are essentially hidden or unavailable. Typically, 20 to 50 oligonucleotides are tested to find one or more active antisense sites per gene.

Standard methods for selecting antisense sites within pre-mRNA or mRNA sequences are insufficient for the rapid, high-throughput application of antisense to large scale target validation programs. Oligonucleotides must be "custom-synthesized" for each target site within each target gene. A standing library of millions or billions of conventional oligonucleotides would be required to successfully target each of the approximately 100,000 human genes. An ordered

library of millions of antisense oligonucleotides is beyond the chemical, physical, and organizational tools currently available.

Summary of the Invention

A new method for preparing and testing antisense and ribozyme sequences has now been invented.

One aspect of the invention is a composition comprising two oligonucleotide analogs, each having a binding domain and a coupling moiety, wherein the binding domains are capable of hybridizing to a target polynucleotide, and the coupling moieties are capable of coupling to each other in the absence of a target molecule.

Another aspect of the invention is a compound of the formula R_1-L_1-X-A-Y-L_2-R_2,

wherein R_1 is an oligonucleotide, or an oligonucleotide analog, capable of binding to RNA; R_2 is an oligonucleotide, or an oligonucleotide analog, capable of binding to RNA; L_1 and L_2 are each independently a linking moiety or a bond; X and Y are each independently a coupling moiety; and A comprises a link selected from the group consisting of a covalent bond, a metal ion, and a non-covalent bond, wherein said compound is capable of activating a nuclease or catalyzing cleavage when bound to a target polynucleotide.

Another aspect of the invention is a method for cleaving a target polynucleotide, comprising providing a target RNA molecule; contacting the target RNA molecule with a first oligonucleotide analog, comprising a first binding domain capable of binding a first region of a target polynucleotide, and a first coupling moiety capable of binding to a second coupling moiety, and a second oligonucleotide analog, comprising a second binding domain capable of binding a second region of said target polynucleotide, and a second coupling moiety capable of binding to said first coupling moiety, wherein said first and second binding domains are capable of binding simultaneously to said target RNA molecule; and incubating said target RNA molecule, first analog and second analog in the presence of an RNase capable of

cleaving the RNA target.

Another aspect of the invention is a method for cleaving a target RNA molecule, comprising providing a target RNA molecule; contacting the target RNA molecule with a first oligonucleotide analog, comprising a first binding domain capable of binding a first region of a target polynucleotide, and a first coupling moiety capable of binding to a second coupling moiety, and a second oligonucleotide analog, comprising a second binding domain capable of binding a second region of said target polynucleotide, and a second coupling moiety capable of binding to said first coupling moiety, wherein said first and second binding domains are capable of binding simultaneously to said target RNA molecule; and incubating said target RNA molecule, first analog and second analog in the presence of an RNase capable of cleaving the RNA target.

Another aspect of the invention is an antisense library, comprising a set of first oligonucleotide analogs, each first analog comprising a first coupling moiety and a first binding domain, said first binding domain comprising a first backbone and a plurality of first bases capable of base-pairing with a target nucleic acid; and a set of second oligonucleotide analogs, each second analog comprising a second coupling moiety capable of coupling specifically to said first coupling moiety, and a second binding domain, said second binding domain comprising a second backbone and a plurality of second bases capable of base-pairing with a target nucleic acid; wherein an antisense analog consisting of a first analog coupled to a second analog is capable of binding to a target nucleic acid and serving as a nuclease substrate.

Another aspect of the invention is a library of antisense precursor compounds, a plurality of compounds of formula 2 (R_1-L_1-X) and a plurality of compounds of formula 3 (Y-L_2-R_2), wherein R_1 and R_2 are each independently an oligonucleotide or an oligonucleotide analog, capable of binding to mRNA; L_1 and L_2 are each independently a linking moiety or a bond; X and Y are each independently a coupling moiety; and wherein said compounds of formula 2 and formula 3 can be coupled to form a compound capable of recruiting or activating a nuclease when bound to a target polynucleotide.

Another aspect of the invention is a library of ribozyme precursor compounds, comprising a plurality of compounds of formula 4 (GG-R_1-CUGAUGA-L_1-X) and a plurality of compounds of formula 5 (Y-L_2-GAA-R_2), wherein R_1 and R_2 are each independently an oligonucleotide or an oligonucleotide analog, capable of binding to RNA; L_1 and L_2 are each independently a linking moiety or a bond; X and Y are each independently a coupling moiety; and wherein said compounds of formula 4 and formula 5 can be coupled to form a ribozyme.

Another aspect of the invention is a method for determining an optimal antisense site for a given mRNA, comprising: selecting a plurality of first oligonucleotide analogs, said first analogs comprising a first coupling moiety and a first binding domain which is complementary to said mRNA; selecting a second oligonucleotide analog for each first oligonucleotide analog, said second analog comprising a second coupling moiety capable of binding said first coupling moiety, and a second binding domain which is complementary to said RNA at a position proximal to the site to which said first binding domain is complementary; coupling said first coupling moieties and said second moieties to provide a plurality of antisense probes; contacting said mRNA with said antisense probes in the presence of an RNase to form a cleavage product; and determining which antisense probe corresponds to said cleavage product.

Another aspect of the invention is a method for determining an optimal ribozyme cleavage site for a given target RNA, comprising: selecting a plurality of first oligonucleotide analogs, said first analogs comprising a first coupling moiety and a first binding domain which is complementary to said target RNA; selecting a second oligonucleotide analog for each first oligonucleotide analog, said second analog comprising a second coupling moiety capable of binding said first coupling moiety, and a second binding domain which is complementary to said RNA at a position proximal to the site to which said first binding domain is complementary; coupling said first coupling moieties and said second moieties to provide a plurality of ribozymes; contacting said target RNA with said ribozymes to form a cleavage product; and determining which ribozyme corresponds to said cleavage product.

One object of the invention is to provide a method for preparing antisense or ribozyme molecules quickly, using a feasible number of pre-synthesized components.

Another object of the invention is to provide a library of components suitable for forming antisense or ribozyme molecules on demand.

Another object of the invention is to provide methods for determining the optimal antisense or ribozyme sequence for a given target.

Another object of the invention is to provide methods for determining an antisense or ribozyme sequence when the target polynucleotide sequence is unknown.

Brief Description of the Drawing

FIG. 1 schematically depicts an oligonucleotide construct of the invention.

FIG. 2 schematically depicts an antisense construct having multiple oligomers.

FIG. 3 illustrates a complex consisting of an anchor oligonucleotide, a cleaver oligonucleotide, and a target RNA.

Detailed Description

Definitions

The term "antisense" as used herein refers to a molecule designed to interfere with gene expression and capable of recognizing or binding to a specific desired target polynucleotide sequence. Antisense molecules typically (but not necessarily) comprise an oligonucleotide or oligonucleotide analog capable of binding specifically to a target sequence present on an RNA molecule. Such binding interferes with translation by a variety of means, including preventing the action of polymerases, RNA processing and recruiting and/or activating nucleases.

The term "ribozyme" as used herein refers to an oligonucleotide or oligonucleotide analog capable of catalytically cleaving a polynucleotide.

The term "oligonucleotide" refers to a molecule consisting of DNA, RNA, or DNA/RNA hybrids.

The term "oligonucleotide analog" refers to a molecule comprising an oligonucleotide-like structure, for example having a backbone and a series of bases, wherein the backbone and/or one or more of the bases can be other than the structures found in naturally-occurring DNA and RNA. "Non-natural" oligonucleotide analogs include at least one base or backbone structure that is not found in natural DNA or RNA. Exemplary oligonucleotide analogs include, without limitation, DNA, RNA, phosphorothioate oligonucleotides, peptide nucleic acids ("PNA"s), methoxyethyl phosphorothioates, oligonucleotides containing deoxyinosine or deoxy 5-nitroindole, and the like.

The term "oligomer" as used herein refers to a component of the invention comprising a binding domain and at least one coupling moiety. The oligomer can be bound to the coupling moiety optionally with a flexible linker. Oligomers can be represented generically by the formula Y-L_1-R-L_2-X, where R is a binding domain, L_1 and L_2 are each independently an optional flexible linker, X is a coupling moiety, and Y is an optional second coupling moiety. Oligomers can further comprise detectable labels. Individual oligomers can be too short to exhibit activity, but are capable of exhibiting activity when coupled.

The term "library" refers to a collection of components that can be joined to form a variety of different antisense molecules. In the practice of the invention, a library comprises at least two sets of oligomers, designed such that oligomers of the first set can couple to oligomers of the second set, preferably spontaneously on addition.

The term "backbone" as used herein refers to a generally linear molecule capable of supporting a plurality of bases attached at defined intervals. Preferably, the backbone will support the bases in a geometry conducive to hybridization between the supported bases and the bases of a target polynucleotide.

The term "unnatural base" refers to a base other than A, C, G, T, and U, and includes degenerate and universal bases as well as moieties capable of binding specifically to a natural base or another unnatural base.

The term "universal base" refers to a moiety that may be substituted for

any base. The universal base need not contribute to hybridization, but should not significantly detract from hybridization. Exemplary universal bases include, without limitation, inosine, 5-nitroindole and 4-nitrobenzimidazole.

The term "degenerate base" refers to a moiety that is capable of base-pairing with either any purine, or any pyrimidine, but not both purines and pyrimidines. Exemplary degenerate bases include, without limitation, 6H,8H-3,4-dihydropyrimido[4,5-c][1,2]oxazin-7-one ("P", a pyrimidine mimic) and 2-amino-6-methoxyaminopurine ("K", a purine mimic).

The term "target polynucleotide" refers to DNA or RNA, for example as found in a living cell, with which the antisense molecule is intended to bind or react.

The term "polarity" as used herein refers to the orientation of a strand or linear molecule. For example, 5'→3' constitutes one polarity, while 3'→5' constitutes an opposite polarity. Not all linear molecules have an inherently defined polarity.

The term "flexible linker" refers to a moiety capable of covalently attaching a binding domain to a coupling moiety. Suitable flexible linkers are typically linear molecules in a chain of at least one or two atoms, more typically an organic polymer chain of 1 to 12 carbon atoms (and/or other backbone atoms) in length. Exemplary flexible linkers include polyethylene glycol, polypropylene glycol, polyethylene, polypropylene, polyamides, polyesters, and the like.

The term "coupling moiety" as used herein refers to a reactive chemical group that is capable of reacting with another coupling moiety to join two molecules. The coupling moieties used in the invention should be able to bind in the absence of any target molecule, and are preferably selected such that the first coupling moiety reacts only with the second coupling moiety (under the conditions under which the library is prepared and used), and not with any other portion of the molecule or other first coupling moieties. Similarly, the second coupling moiety should react only with the first coupling moieties, and not with any other second coupling moiety (or any other portion of the molecules). Exemplary coupling moieties include complementary oligonucleotides (preferably selected such that they do not hybridize to any portion of the target polynucleotide), complementary oligonucleotide analogs (particularly

employing bases which do not hybridize to natural bases), and electrophilic or nucleophilic moieties such as alkyl halides, alkyl sulfonates, activated esters, ketones, aldehydes, amines, hydrazines, sulfhydryls, alcohols, phosphates, thiophosphates, Michael addition receptors, dienophiles, dienes, dipolarophiles, nitriles, thiosemicarbazides, imidates, isocyanates, isothicyanates, alkynes, and alkenes. Where the antisense constructs comprise more than two component parts (for example, where three or four molecules are coupled to make the final construct), the coupling moieties are preferably selected such that the first and second coupling moieties react only with each other, and the third and fourth coupling moieties react only with each other, and so forth.

The term "stem" as used herein refers to the structure formed by coupling two oligonucleotide or oligonucleotide analog coupling moieties.

The term "activity" refers to the ability of an antisense molecule of the invention, when hybridized to a target polynucleotide, to interfere with the transcription and/or translation of the target polynucleotide. Preferably, the interference arises because the antisense molecule when hybridized serves to recruit a nuclease, and/or serves as a nuclease substrate. "Interference" includes inhibition to any detectable degree.

The term "hydrocarbyl" refers to a moiety consisting of carbon and hydrogen, and containing from one to about twelve carbon atoms. Exemplary hydrocarbyl groups include, without limitation, methyl, ethyl, propyl, butyl, 2-butyl, t-butyl, hexyl, and the like.

General Method

A preformed library of oligonucleotide analogs is provided, comprising a set of first oligonucleotide analogs and a set of second oligonucleotide analogs, the analogs having coupling moieties that provide for coupling each first oligonucleotide analog to a second oligonucleotide analog to form an antisense molecule. The oligonucleotide analogs are selected to act, when coupled, as a substrate for an endonuclease that recognizes double-stranded (ds) RNA or RNA/DNA hybrids when

hybridized to a target nucleic acid. The binding domains need to be long enough to insure that the antisense molecule binds to the target polynucleotide, and is able to recruit and/or activate a nuclease. However, the number of molecules required for a complete library increases exponentially with the length of the sequence represented.

By conceptually separating the antisense molecules into two or more pieces, a comprehensive antisense library can be prepared in advance, rather than synthesizing a plurality of candidate antisense molecules as needed. A complete library of every possible 17mer oligonucleotide, using the four natural bases, would consist of 4^{17} (or about 1.7×10^{10}) molecules. By providing the antisense molecules in at least two components, for example a library of 8mers and a library of 9mers, assembled quickly as needed, the size of the library needed is reduced to $4^8 + 4^9$, or 327,650 molecules. The required complexity of the library is still further reduced by substituting one or more universal or degenerate bases for some of the natural bases. Thus, for example, if the 9mer library consists of 5 universal bases followed by 4 natural bases, the number of components drops to 4^4 (256), and the total library size is reduced to $4^8 + 4^4$, about 66,000 molecules. The library complexity can also be reduced by dividing the antisense molecule into three or more segments. For example, a full 18mer library would require 4^{18} molecules, or about 6.9×10^{11} molecules. However, a library composed of first, second, and third hexamers that assemble to form 18mers need only contain $4^6 + 4^6 + 4^6$, or 12,288 molecules, a size attainable with current parallel synthesis technology. If, for example, the middle hexamer set is replaced with a hexamer of universal bases, the library complexity is reduced by a third. It is possible to synthesize and maintain libraries of this size, and rapidly assemble any desired antisense molecule without the need for custom, de novo synthesis of long oligomers. Thus, one embodiment of the invention is a library comprising at least two sets of oligomers, wherein oligomers are selected from each set and coupled as needed.

The library size can be further reduced by avoiding certain sequences which are predicted to serve as poor antisense molecules by reason of poor binding ability, for example, AT-rich molecules; or artifact formation, for example, AG-rich

regions, poly-C, $(GGN)_n$, $(GGGN)_n$, and TAT motifs.

FIG. 1 is a schematic illustration of an RNA-analog complex 100, having RNA 101 hybridized to an oligonucleotide analog comprising a first "anchor" domain 102 and a second "cleaver" domain 103 which hybridize to adjacent regions 110 and 111 of the RNA. The "cleaver" domain is also able to serve as a nuclease substrate. The first and second domains are connected to each other through first and second coupling moieties 106 and 107, which are linked to the first and second binding domains 102 and 103 by flexible linkers 104 and 105, respectively.

FIG. 2 depicts an RNA-antisense molecule complex 200 having multiple oligomers. Binding domains 205, 206, 207 are coupled together by coupling moieties, here complementary oligonucleotides 208, 209, 210, 211 which are joined to the binding domains by flexible linkers 212. Target regions 202, 203, 204 can be adjacent, contiguous, or slightly spaced apart. The binding domains need not be of equal length.

At least one of the binding domains comprises about 3 to about 24 bases, preferably about 6 to about 8 bases. If desired, one domain may provide most of the target specificity, while the other domain primarily provides a nuclease substrate. For example, a library can be constructed having a set of 6mer binding domains, each of which binds only a single 6mer sequence, and a set of 8mer binding domains, in which only four of the bases are sequence-specific, and the remaining bases are degenerate or universal. The first set contains a possible 4^6 (4096) sequences (prior to eliminating undesirable sequences), while the second set contains only 4^4 (256) sequences (prior to eliminating undesirable sequences, assuming 4 "specific" bases and 4 universal bases). By combining oligomers selected from the first and second sets as needed, one can generate 4^{10} (10^6) different sequences using only 4,352 molecules. In contrast, a complete library of 14mers would require 4^{14} (2.7×10^8) molecules.

The oligomers can be synthesized using standard oligonucleotide synthesis methods. For example, one can employ combinatorial synthesis techniques using pooling and splitting methods with AccuTag reactors (Irori, La Jolla, CA).

Oligonucleotides can be synthesized on solid supports and stored, or cleaved into solution and stored until required. The oligonucleotides can be synthesized attached to solid supports by cleavable linkers. If desired, one can use linkers that can be cleaved under cell culture conditions (i.e., the linkers can be cleaved in the presence of cells without damaging the cells). Suitable linkers include photolabile linkers (Glen Research), linkers cleaved by β-elimination, oxidative cleavage, and enzymatic activity (for example, RNases, esterases, proteases and the like). This permits one to store and dispense the oligomers in a dry state, coupling them *in situ*.

The oligomer synthesis can be performed in the 3' to 5' direction for some library components, and the 5' to 3' direction for other components. In cases where the coupling moieties are oligonucleotides or oligonucleotide analogs, it is preferable to synthesize the coupling moiety last, so that synthesis failures result in molecules having an incomplete stem (and thus unable to couple).

The oligomers used in the binding domains can employ any backbone capable of resulting in a molecule that hybridizes to natural nucleic acids (DNA and/or RNA). Examples of suitable backbones include phosphodiesters and deoxy phosphodiesters, phosphorothioates and deoxy phosphorothioates, 2'-O-substituted phosphodiesters and deoxy analogs, 2'-O-substituted phosphorothioates and deoxy analogs, morpholino, peptide nucleic acids (Nielsen et al., US 5,539,082), 2'-O-alkyl methylphosphonates, 3'-amidates, MMI, alkyl ethers (Cook et al., US 5,223,618) and others as described in Cook et al., US 5,378,825, Sanghvi et al., US 5,489,677, Cook et al., US 5,541,307, and the like. Where RNase activity is desired, a backbone capable of serving as an RNase substrate is employed for at least a portion of the oligomer.

Suitable bases include the following, without limitation:

Nucleoside base	cleaver/anchor / stem	complexity (pairs with _)	Commercial?
deoxy adenosine	cleaver	normal	yes
deoxy guanosine	cleaver	normal	yes
deoxy cytidine	cleaver	normal	yes

Nucleoside base	cleaver/anchor / stem	complexity (pairs with _)	Commercial?
thymidine	cleaver	normal	yes
deoxy diaminopurine	cleaver	normal, (U)	yes
deoxy propynyl C	cleaver	normal, (G)	yes
deoxy propynyl U	cleaver	normal, (A)	yes
deoxy 5-nitroindole	cleaver	universal	yes
deoxy P	cleaver	generic (A&G)	yes
deoxy K	cleaver	generic (U&C)	yes
deoxy 3-nitropyrrole	cleaver	universal	yes
deoxy 4-nitrobenzimidazole	cleaver	universal	
deoxy nebularine	cleaver	universal	yes
deoxy inosine	cleaver	universal	Yes
deoxy 2-aminopurine	cleaver	generic (U&C)	yes
2'-OMe adenosine	anchor	normal	yes
2'-OMe guanosine	anchor	normal	yes
2'-OMe cytidine	anchor	normal	yes
2'-OMe uridine	anchor	normal	yes
2'-OMe diamino purine	anchor/stem	normal, (U)	yes
2'-OMe inosine	anchor	universal	Yes
2'-OMe 2-aminopurine	anchor	generic (U&C)	yes
2'-OMe nebularine	anchor	universal	
2'-OMe 5-nitroindole	anchor	universal	
2'-OMe propynyl C	anchor/stem	normal	yes
2'-OMe propynyl U	anchor/stem	normal	yes
2'-OMe P	anchor	generic (G&A)	
2'-OMe K	anchor	generic (U&C)	

Nucleoside base	cleaver/anchor / stem	complexity (pairs with _)	Commercial?
2'-OMe 4-nitrobenzimidazole	anchor	universal	
2'-OMe 3-nitropyrrole	anchor	universal	
2'-F adenosine	anchor	normal	yes
2'-F guanosine	anchor	normal	yes
2'-F cytidine	anchor	normal	yes
2'-F uridine	anchor	normal	yes
2'-F diaminopurine	anchor/stem	normal (U)	
2'-F inosine	anchor	universal	
2'-F-2-amino purine	anchor/stem	generic (U&C)	
2'-F nebularine	anchor	universal	
2'-F 5-nitroindole	anchor	universal	
2'-F propynyl C	anchor/stem	normal	
2'-F propynyl U	anchor/stem	normal	
2'-F P	anchor	generic (G&A)	
2'-F K	anchor	generic (U&C)	
2'-F 4-nitrobenzimidazole	anchor	universal	
2'-F 3-nitropyrrole	anchor	universal	
PNA-A	anchor/stem	normal	yes
PNA-G	anchor/stem	normal	yes
PNA-C	anchor/stem	normal	yes
PNA-T	anchor/stem	normal	yes
PNA-5-nitroindole	anchor	universal	
PNA propynyl C	anchor/stem	normal	
PNA-propynyl U	anchor/stem	normal	
PNA-2-aminopurine	anchor	generic (U&C)	

Nucleoside base	cleaver/anchor / stem	complexity (pairs with _)	Commercial?
PNA-diaminopurine	anchor/stem	normal	
PNA-nebularine	anchor	universal	
PNA-inosine	anchor	universal	
PNA-P	anchor	generic (G&A)	
PNA-K	anchor	generic (U&C)	
PNA-4-nitrobenzimidazole	anchor	universal	
PNA-3-nitropyrrole	anchor	universal	
morpholino-A	anchor/stem	normal	yes
morpholino-G	anchor/stem	normal	yes
morpholino-C	anchor/stem	normal	yes
morpholino-U	anchor/stem	normal	yes
morpholino-5-nitroindole	anchor	universal	
morpholino-propynyl C	anchor/stem	normal	
morpholino-propynyl U	anchor/stem	normal	
morpholino-2-aminopurine	anchor	generic (U&C)	
morpholino-diaminopurine	anchor/stem	normal	
morpholino-nebularine	anchor	universal	
morpholino-inosine	anchor	universal	
morpholino-P	anchor	generic (G&A)	
morpholino-K	anchor	generic (U&C)	
morpholino-4-nitrobenzimidazole	anchor	universal	
morpholino-3-nitropyrrole	anchor	universal	
phosphoramidate-A	anchor/stem	normal	yes
phosphoramidate-C	anchor/stem	normal	yes
phosphoramidate-G	anchor/stem	normal	yes

Nucleoside base	cleaver/anchor / stem	complexity (pairs with _)	Commercial?
phosphoramidate-U	anchor/stem	normal	yes
phosphoramidate-5-nitroindole	anchor	universal	
phosphoramidate-propynyl C	anchor/stem	normal	
phosphoramidate-propynyl U	anchor/stem	normal	
phosphoramidate-2-aminopurine	anchor	generic (C&U)	
phosphoramidate-diaminopurine	anchor/stem	normal	
phosphoramidate-nebularine	anchor	universal	
phosphoramidate-inosine	anchor	universal	
phosphoramidate-P	anchor	generic (G&A)	
phosphoramidate-K	anchor	generic (U&C)	
phosphoramidate-4-nitrobenzimidazole	anchor	universal	
phosphoramidate-3-nitropyrrole	anchor	universal	
2'-O-methoxyethyl adenosine	anchor	normal	
2'-O-methoxyethyl guanosine	anchor	normal	
2'-O-methoxyethyl cytidine	anchor	normal	
2'-O-methoxyethyl uridine	anchor	normal	
2'-O-methoxyethyl diaminopurine	anchor/stem	normal (U)	
2'-O-methoxyethyl inosine	anchor	universal	
2'-O-methoxyethyl 2-aminopurine	anchor	generic (U&C)	
2'-O-methoxyethyl nebularine	anchor	universal	
2'-O-methoxyethyl 5-nitroindole	anchor	universal	
2'-O-methoxyethyl propynyl C	anchor/stem	normal	
2'-O-methoxyethyl propynyl U	anchor/stem	normal	
2'-O-methoxyethyl P	anchor	generic (G&A)	

Nucleoside base	cleaver/anchor / stem	complexity (pairs with _)	Commercial?
2'-O-methoxyethyl K	anchor	generic (U&C)	
2'-O-methoxyethyl 4-nitro-benzimidazole	anchor	universal	
2'-O-methoxyethyl 3-nitropyrrole	anchor	universal	
deoxy Rp MP-AG dimer	anchor/stem	normal	
deoxy Rp MP-GA dimer	anchor/stem	normal	
deoxy Rp MP-AC dimer	anchor/stem	normal	
deoxy Rp MP-CA dimer	anchor/stem	normal	
deoxy Rp MP-AT dimer	anchor/stem	normal	
deoxy Rp MP-TA dimer	anchor/stem	normal	
deoxy Rp MP-AA dimer	anchor/stem	normal	
deoxy Rp MP-GG dimer	anchor/stem	normal	
deoxy Rp MP-CC dimer	anchor/stem	normal	
deoxy Rp MP-TT dimer	anchor/stem	normal	
deoxy Rp MP-GC dimer	anchor/stem	normal	
deoxy Rp MP-CG dimer	anchor/stem	normal	
deoxy Rp MP-GT dimer	anchor/stem	normal	
deoxy Rp MP-TG dimer	anchor/stem	normal	
deoxy Rp MP-CT dimer	anchor/stem	normal	
deoxy Rp MP-TC dimer	anchor/stem	normal	
deoxy Rp MP-5-nitroindole dimer	anchor	universal	
deoxy Rp MP-KP dimer	anchor	generic	
deoxy Rp MP-PK dimer	anchor	generic	
deoxy Rp MP-KK dimer	anchor	generic	
deoxy Rp MP-PP dimer	anchor	generic	

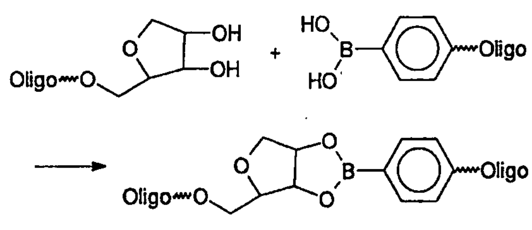
Nucleoside base	cleaver/anchor / stem	complexity (pairs with _)	Commercial?
2'-OMe Rp MP-AG dimer	anchor/stem	normal	
2'-OMe Rp MP-GA dimer	anchor/stem	normal	
2'-OMe Rp MP-AC dimer	anchor/stem	normal	
2'-OMe Rp MP-CA dimer	anchor/stem	normal	
2'-OMe Rp MP-AT dimer	anchor/stem	normal	
2'-OMe Rp MP-TA dimer	anchor/stem	normal	
2'-OMe Rp MP-AA dimer	anchor/stem	normal	
2'-OMe Rp MP-GG dimer	anchor/stem	normal	
2'-OMe Rp MP-CC dimer	anchor/stem	normal	
2'-OMe Rp MP-TT dimer	anchor/stem	normal	
2'-OMe Rp MP-GC dimer	anchor/stem	normal	
2'-OMe Rp MP-CG dimer	anchor/stem	normal	
2'-OMe Rp MP-GT dimer	anchor/stem	normal	
2'-OMe Rp MP-TG dimer	anchor/stem	normal	
2'-OMe Rp MP-CT dimer	anchor/stem	normal	
2'-OMe Rp MP-TC dimer	anchor/stem	normal	
2'-OMe Rp MP-5-nitroindole dimer	anchor	universal	
2'-OMe Rp MP-KP dimer	anchor	generic	
2'-OMe Rp MP-PK dimer	anchor	generic	
2'-OMe Rp MP-KK dimer	anchor	generic	
2'-OMe Rp MP-PP dimer	anchor	generic	
RiboPyranoysl A	stem	self/normal	
RiboPyranoysl G	stem	self/normal	
RiboPyranoysl C	stem	self/normal	
RiboPyranoysl U	stem	self/normal	

Nucleoside base	cleaver/anchor / stem	complexity (pairs with _)	Commercial?
MMI-AG dimer	anchor/stem	normal	
MMI-GA dimer	anchor/stem	normal	
MMI-AC dimer	anchor/stem	normal	
MMI-CA dimer	anchor/stem	normal	
MMI-AT dimer	anchor/stem	normal	
MMI-TA dimer	anchor/stem	normal	
MMI-AA dimer	anchor/stem	normal	
MMI-CC dimer	anchor/stem	normal	
MMI-GG dimer	anchor/stem	normal	
MMI-TT dimer	anchor/stem	normal	
MMI-GC dimer	anchor/stem	normal	
MMI-CG dimer	anchor/stem	normal	
MMI-GT dimer	anchor/stem	normal	
MMI-TG dimer	anchor/stem	normal	
MMI-CT dimer	anchor/stem	normal	
MMI-TC dimer	anchor/stem	normal	

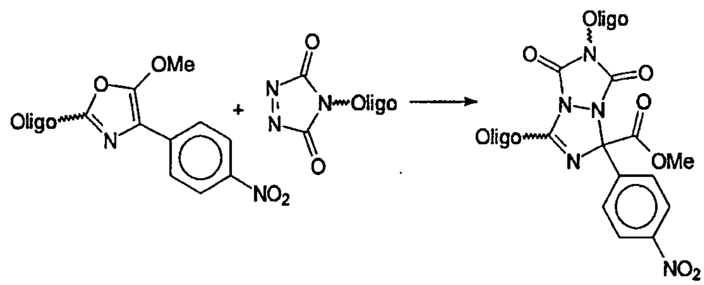
The coupling moieties are selected to join two oligomers from different sets by either covalent or non-covalent interaction, for example a non-covalent binding pair. The coupling moieties are preferably selected such that the coupling moiety present on oligomers of one set in a library do not couple with each other, but bind readily with coupling moieties on oligomers of the another set, thus insuring that the oligomers couple in the intended orientation. In one embodiment, the coupling moieties are complementary oligonucleotides. The complementary regions can be separated by several non-complementary bases, to provide an inherent flexible linker. The complementary oligonucleotides can be attached to the binding domains in the same polarity or orientation, or can be provided in reverse polarity or orientation. For

example, where the binding domain is in the 5'-3' orientation, the complementary oligonucleotide coupling moiety can be attached in the 3'-5' orientation, thus reducing the chances that the coupling moiety will inadvertently participate (or interfere with) binding to the target polynucleotide. In another embodiment, the oligonucleotide comprises unnatural bases which do not hybridize with natural bases.

The coupling moieties may also join as the result of covalent chemical interactions, for example, by condensation, cycloaddition, or nucleophilic-electrophilic addition. In one embodiment, one coupling moiety can be a sulfhydryl group, while its complementary coupling moiety is a succinimidyl group. In another embodiment, one coupling moiety is an amine or a hydrazine moiety, while the complementary coupling moiety is a carbonyl group (aldehyde, ketone, or activated ester). In another embodiment, one coupling moiety is a maleimidyl group while the complementary coupling moiety is a sulfhydryl group. In another embodiment, one coupling moiety is an aryl-dihydroxyboron group which binds to adjacent OH groups on ribose.



In another embodiment, an oxazole derivative forms one coupling moiety, while its complement comprises a diketotriazole, as described by T. Ibata et al., Bull Chem Soc Japan (1992) 65:2998-3007:



In libraries with more than two sets of oligomers (i.e., where the antisense molecule comprises three or more oligomers coupled together), the coupling moieties can be selected to be orthogonal to each other to insure that the oligomers are assembled in the intended order. For example, the coupling moieties between the first and second oligomers can be sulfhydryl and maleimide groups, while the coupling moieties between the second and third oligomers can be diene and dienophile groups. Suitable coupling moieties include, without limitation, alkyl halides, alkyl sulfonates, activated esters, ketones, aldehydes, amines, hydrazines, sulfhydryls, alcohols, phosphates, thiophosphates, Michael addition receptors, dienophiles, dienes, dipolarophiles, nitriles, alkynes, thiosemicarbazides, isothiocyanates, isocyanates, imidates, and alkenes.

Flexible linkers are optionally used to relieve stress that might otherwise result from interposing the coupling moieties between two binding domains that bind to adjacent regions of target nucleic acid. The flexible linker is preferably selected to be flexible, hydrophilic, and of sufficient length that the bulk of the coupling moieties does not interfere with hybridization, RNase recognition, and/or RNase activity on the complex. It is preferred, but not essential, to employ a flexible linker between each

binding domain and its coupling moiety. It is preferred to employ a linker at least between the binding domain and coupling moiety that serves as an RNase substrate, and more preferred to employ flexible linkers in each oligomer. The linker may be connected to the terminal base of the binding domain, or can be connected one or more bases from the end. Suitable flexible linkers are typically linear molecules in a chain of at least one or two atoms, more typically an organic polymer chain of 1 to 12 carbon atoms (and/or other backbone atoms) in length. Flexible linkers also include additional bases, not complementary to the target sequence. Exemplary flexible linkers include polyethylene glycol, polypropylene glycol, polyethylene, poly-propylene, polyamides, polyesters, and the like.

The individual oligomers can be assembled in vitro or in vivo. The coupling moieties are preferably selected to join spontaneously, under the conditions of the intracellular environment. Thus, one can administer separate oligomers individually, for agent formation in vivo. Alternatively, one can join oligomers in vitro prior to administration. One can further employ transfection aids to increase the rate of uptake, for example Lipofectin, Lipofectamine, Lipofectace, and the like.

The activity of various constructs of the invention can be determined by standard assay methods, for example as set forth in the Examples below. In general, one can prepare a target polynucleotide having a known sequence, contact the target with oligomers of the invention selected to bind the target sequence to form a complex, subject the complex to cleavage with the desired target nuclease, and analyze the products to determine if cleavage occurred.

The library of the invention can be prepared in advance. If a sequence for a target polynucleotide is supplied, oligomers corresponding to the sequence are selected from the library, combined to form a plurality of antisense agents, and the agents applied to a plurality of test cells that express the target. The agents can be applied individually or in mixtures. Activity can be determined by detecting cleaved target polynucleotides directly (e.g., by hybridization to a labeled probe, amplification with PCR, visualization on a gel, and the like), or by an effect on the host cell phenotype (for example, expression or lack of expression of a selected protein).

Alternatively, where the target sequence is unknown, one can assemble a plurality of agents and determine empirically which sequences result in active agents. For example, assume a protein of unknown sequence expressed by a known cell. One can provide a plurality of antisense molecules of the invention consisting of every combination of oligomers in each set in the library, e.g.,

$Oligo_1a$-$Oligo_2a$	$Oligo_1a$-$Oligo_2b$	$Oligo_1a$-$Oligo_2c$	...
$Oligo_1b$-$Oligo_2a$	$Oligo_1b$-$Oligo_2b$	$Oligo_1b$-$Oligo_2c$	...
$Oligo_1c$-$Oligo_2a$	$Oligo_1c$-$Oligo_2b$	$Oligo_1c$-$Oligo_2c$	...
$\vdots$	$\vdots$	$\vdots$	$\ddots$

As there may be too many antisense molecules to investigate each combination individually, it may be preferable to pool the antisense molecules for testing. For example, if the first set contains about 4,000 hexamer sequences (all bases non-degenerate and non-universal), and the second set contains about 250 octamer sequences (having 4 universal bases and 4 specific bases), the complete library would contain about 10^6 individual combinations. These molecules can be pooled easily, for example by coupling each individual octamer sequence to a mixture of all hexamer sequences, resulting in 250 pools of 4,000 combinations. Each pool is then tested against cells known to express the unidentified protein, and pools that result in modulation of the protein expression are identified. The active pools can be further subdivided (for example, by coupling the "active" octamer with 200 individual mixtures of hexamer, to form 200 pools of 200 combinations) and tested iteratively until the active antisense molecule is identified, or examined by other means.

The oligomers and methods of the invention can also be applied to generate ribozymes, and libraries of ribozymes. The minimum sequence requirement for ribozyme activity are described by F. Benseler et al., J Am Chem Soc (1993) 115:8483-84, incorporated herein by reference. Hammerhead ribozyme molecules comprise end domains ("I" and "III") which hybridize to the substrate polynucleotide, a catalytic portion, and a stem loop structure ("II") which can be substituted by a variety of other structures capable of holding the molecule together. These molecules

can be assembled from oligomers of the invention, replacing the II domain stem loop with coupling moieties as described above. Thus, for example, one can prepare a library of oligomers having as the first set a plurality of oligonucleotides or oligonucleotide analogs having the sequence 5'-GGNNNNNCUGAUGA-cp_1 (SEQ ID NO:1) (domain I, first portion of catalytic moiety, and first coupling moiety), and as the second set a plurality of oligonucleotides or oligonucleotide analogs having the sequence 5'-cp2-GAANNNNN (SEQ ID NO:2) (second coupling moiety, second portion of catalytic moiety, and domain III), where the bases "N" are selected to hybridize to the target substrate. These ribozymes can be assembled in advance, for determining the optimum cleavage site of a target polynucleotide of known sequence, or can be assembled in a combinatorial fashion as described above, to determine effective molecules for inhibiting a target of unknown sequence.

Examples

The following examples are provided as a guide for those skilled in the art, and are not to be construed as limiting the invention in any way. All products are used according to manufacturer's instructions, and experiments are conducted under standard conditions, unless otherwise specified.

All reagents are dry (<30 ppm water). DNA synthesis reagents (oxidizer, tetrazole, capping reagents, propyl linker support, 2'-deoxy, 2'-OMe, and spacer 9 amidites) were purchased from Glen Research (Sterling, VA). Amidites in solution are dried over Trap-paks from Perseptive Biosystems. Protected amino acids, PyBOP, and chlorotritylchloride resin were obtained from NovaBiochem.

Example 1

(Cleaver Synthesis-Hybridization Motif)

(A) A solid support that was previously derivatized with a dimethoxy trityl group (DMT) protected propyl linker was placed in a DNA synthesizer column compatible with a Perseptive Expedite synthesizer (1 μmole of starting propyl linker). The DMT group was removed with a deblock reagent (2.5% dichloroacetic acid in

dichloromethane (DCM)). The standard protocols for RNA synthesis were applied to 2'-OMe β-cyanoethyl amidites (0.1 M in dry acetonitrile). The amidites were activated with tetrazole (0.45 M in dry acetonitrile). Coupling times were typically up to 15 minutes for 2'-OMe amidites. To synthesize the stem portion of the cleaver oligonucleotides, the 2'-OMe phosphonite intermediate was treated with an oxidizer (0.02 M iodine in THF/pyridine/water 68/20/2). After each oxidation step, a capping step which placed an acetyl group on any remaining uncoupled 5'-OH groups was introduced by treatment with a mixture of two capping reagents (CAP A = acetic anhydride in THF, and CAP B = N-methylimidazole in THF). The cycle was repeated 15 times with various amidites to obtain the desired sequence. Spacer 9 (Glen Research, cat# 10-1909-90) was introduced using manual coupling protocols. Spacer 9 was coupled twice to ensure proper coupling. Manual coupling was done by attaching the column containing the support with the first part of the oligonucleotide to a syringe containing deblock solution. Solution was passed until all orange color disappeared. The column was washed with dry acetonitrile (3 x 10 ml). One syringe containing 100 μl of activator was attached to one end of the column and another syringe containing 100 μl of the amidite (0.1 M solution) was attached to the other end of the column. The syringes were plunged alternately to drive the mixture of activator and amidite back and forth over the support. The procedure for coupling the amidite was repeated. The support was then washed with acetonitrile (10 ml) and the support treated with oxidizer solution (3 ml). The support was then washed again with acetonitrile (10 ml) and capping solution (an equal mixture of cap A and cap B, freshly mixed) was passed over the support. The support was washed with dry acetonitrile (10 ml). The trityl group of the spacer 9 was removed with deblock solution, and the support washed with acetonitrile (10 ml) before placing the column on the synthesizer. A segment of deoxyphosphorothioate was then synthesized. This was done by coupling 2'-deoxy-β-cyanoethyl phosphoramidites to the spacer 9 linker. The standard coupling cycle of the Expedite was used. The exception to the cycle described above for 2'-OMe was that coupling times were typically shorter and Beaucage sulfurizing reagent (Glen Research, cat.# 40-4036-10) was used instead of

iodine oxidizer to give the phosphorothioate internucleotide linkage. The trityl group was allowed to remain on the last base. The support was treated at 55° in concentrated NH_4OH for 16 hours. The solution was concentrated on a speed vac and the residue taken up in 100 µl aqueous 0.1 M triethyl ammonium acetate ("TEAA"). This was applied to an HPLC column (C-18, Kromasil, 5 µm, 4.3 mm diameter, 250 mm length) and eluted with a CH_3CN gradient (solvent A: 0.1 M TEAA, solvent B: 0.1 M TEAA and 50% acetonitrile) over 30 minutes at 1 ml/min. flow rate. Fractions of greater than 80% pure product were pooled and concentrated. The resulting residue was taken up in 80% acetic acid in water to remove the trityl group and reapplied to a reverse phase column and purified as described above. Fractions containing greater than 90% purity were pooled and concentrated.

(B) Oligonucleotides useful for recruiting RNase L are prepared as in part (A) above, substituting 2'-OMe phosphoramidites for the deoxy amidites used after spacer 9. The resulting oligo has a 2'-OMe diester portion at the 3' side of the spacer, and a 2'-OMe phosphorothioate on the 5' side of the spacer. A linker attached to oligo 2'-5' adenosine is attached to the 5' end of the oligo as described by Torrence et al., US 5,583,032, and US 5,677,289, both incorporated herein by reference. The product is purified as described by Torrence et al.

(C) Rhodamine Labelled Cleaver: A rhodamine labelled cleaver was synthesized as in part (A) above, except that the trityl group was removed from the last base and to that base was coupled a protected amine linker (Perkin-Elmer cat. # 402872). The deprotection of the oligonucleotide was performed as described in part (A) above. The oligonucleotide was taken up in 10 M NH_4OAc and EtOH added to make a 70% ethanolic solution to precipitate the oligonucleotide. The oligonucleotide was pelleted, and the pellet taken up in 100 mM $NaHCO_3$. The isothiocyanate derivative of rhodamine (Molecular Probes cat. # X-491) was added and the mixture allowed to stir for 4 hours. The mixture was purified as described in part (A) above.

Example 2

(Anchor Synthesis-Hybridization Motif)

(A) An oligonucleotide was prepared as described in Example 1(A) above, except that the 2'-OMe amidites that are added before the spacer ("9") (synthesizing 3'-5') are oxidized with Beaucage reagent to form phosphorothioate linkages. 2'-OMe amidites are used after the spacer 9 linkage and are oxidized with the iodine oxidizer to give phosphodiester linkages. The resulting oligonucleotide was purified as in Example 1.

(B) Fluroescein Labelled Anchor: A fluorescein-labelled anchor was synthesized as in part (A) above, except that the trityl group was removed from the last base and to that base was coupled a protected amine linker (Perkin-Elmer cat. # 402872). The deprotection of the oligonucleotide was done as described in part (A). The oligonucleotide was taken up in 10 M NH_4OAc and EtOH added to make a 70% ethanolic solution to precipitate the oligonucleotide. The oligonucleotide was pellet was taken up in 100 mM $NaHCO_3$. The isothiocyanate derivative of fluorescein (Molecular Probes cat. # F-1907) was added and the mixture allowd to stir for 4 hours. The mixture was purified as described in part (A).

Example 3

(Cleavers with Pyranosyl RNA Stems)

Cleaver oligonucleotides were prepared as described in Example 1 above, but substituting pyranosyl RNA monomers for the 2-OMe β-cyanoethyl amidites. Synthesis and deprotection conditions were used as described by Pitsch et al., Helv Chimica Acta (1993) 76:2161-2183.

Example 4

(Anchors with Pyranosyl RNA Stems)

Anchor oligonucleotides were prepared as described in Example 2 above, but substituting pyranosyl RNA monomers and synthesis conditions after the spacer 9 linker for the 2'-OMe monomers and phosphodiester synthesis conditions. Purification was as described in Example 2.

Example 5

(Cleaver Synthesis-Hybridization Motif)

Histidine 6 Synthesis: To a suspension of 2-chlorotritylchloride resin in dry CH_2Cl_2 (DCM) was added N-α-Fmoc-N-π-t-butoxymethyl-L-histidine (0.6 eq, Fmoc-His(Bum)-OH) with sufficient dimethylacetamide to provide solubility. Diisopropylamine (4 eq) was added, and the mixture stirred strongly for 30 min. The product was filtered, and the resin washed with 3X DCM/MeOH/DIPEA) (17:2:1), followed by 3 DCM washes, 2 DMF washes, 2 more DCM washes, and finally 2 MeOH washes. The resin was dried in vacuo over KOH to remove excess MeOH. Loading of histidine was determined spectrophotometrically by release of Fmoc with 20% piperidine in DMF. The first Fmoc was removed by treating the Fmoc-histidine resin with 5% piperidine in DCM/DMA (1:1) for 10 min, followed by 20% piperidine in DMA for 15 min. The free amine was treated with Fmoc-His(Bum)-OH (2.5 eq), PyBOP (2.5 eq), and DIPEA (5 eq) in DMA. Coupling was allowed to proceed for 30 min, after which the resin was filtered and washed with DMA. The Fmoc was removed again with 20% piperidine in DMA, and the coupling cycle repeated three more times to provide a resin-bound His hexamer. The hexamer was removed from the resin and the Bum protecting groups removed with 95% aqueous trifluoroacetic acid. The product was purified by RP-HPLC (Kromasil C18, 5 μm, 4.3 mm diameter, 250 mm length) with a gradient from solvent A to solvent B of 50 min (A: 0.1% TFA/H_2O; B: CH_3CN/H_2O/TFA 90/10/0.1). Fractions of >90% purity were pooled and concentrated by speed vac. The structure was confirmed by positive ion mass spectroscopy [M+H] 802.

Synthesis of Z-ACPID: Z-(amino-1-carboxypentyl)iminodiacetic acid (Z-ACPID) was synthesized according to the procedure of Hochuli et al., J Chromatog (1987) 411:177-84.

Synthesis of ACPID: A suspension of Z-protected NTA (2.0 g) in 1:3 H_2O:EtOH was heated until the mixture became clear. To this was added 10% Pd/C (2.0 g) and 20 ml of cyclohexene. The mixture was heated at reflux for 2 hours. The Pd/C was filtered, and the filtrate reduced in vacuo to give a solid foam. The structure was confirmed by negative ion mass spectroscopy [M-H] 261. Yield was 900 mg.

ACPID Derivatized Cleaver: A solid support that was previously derivatized with a dimethoxy trityl group (DMT) protected propyl linker is placed in a DNA synthesizer column compatible with a Perseptive Expedite synthesizer (1 μmole of starting propyl linker). The DMT group is removed with a deblock reagent (2.5% dichloroacetic acid in dichloromethane). The standard protocols for DNA synthesis are applied to 3'O-DMT-5'-O-β-cyanoethyl amidites (0.1 M in dry acetonitrile, <30 ppm H_2O). The amidites are activated with tetrazole (0.45 M in dry acetonitrile, <30 ppm H_2O). The phosphonite intermediate is treated with Beaucage reagent to form the phosphorothioate linkage. After each oxidation step a capping step which places an acetyl group on any remaining uncoupled 3'-OH groups is introduced by treatment with a mixture of two capping reagents (CAP A:acetic anhydride in THF and CAP B:N-methylimidazole in THF). The cycle is repeated 12 times with various bases to obtain the desired sequence. To the last 3'-OH is coupled a thiol-modifier (thiol modifier C6 S-S, Glen Research 10-1936) that puts a protected disulfide on the oligo-nucleotide. The DMT is removed with DCA. The support is treated with excess tris-carboxyethyl phosphine (TCEP, Pierce cat. # 20490) and washed to remove the excess TCEP. The resulting sulfhydryl is treated with excess succinimidyl 4-(p-maleimidophenyl)butyrate (SMPB, Pierce cat. # 22315). The support is washed to remove excess SMPB. The resulting NHS ester is reacted with excess ACPID ((amino-1-carboxypentyl)iminodiacetic acid). The resulting metal chelating oligonucleotide conjugate is then washed to remove excess ACPID. The support is

treated at 55 °C in concentrated ammonium hydroxide for 16 hours. The solution is concentrated on a speed vac and the residue taken up in 100 μl aqueous 0.1 M triethyl ammonium acetate. This is applied to an HPLC column (C-18, Kromasil, 5 μm, 4.3 mm diameter, 250 mm length) and eluted with an acetonitrile gradient (solvent A: 0.1 M TEAA; solvent B: 0.1 M TEAA and 50% acetonitrile) over 30 minutes at 1 ml/min. flow rate. Fractions of greater than 90% purity are pooled and concentrated.

<u>His 6 Derivatized Anchor</u>: A solid support that was previously derivatized with a dimethoxy trityl group (DMT) protected propyl linker is placed in a DNA synthesizer column compatible with a Perseptive Expedite synthesizer (1 μmole of starting propyl linker). The DMT group is removed with a deblock reagent (2.5% dichloroacetic acid in dichloromethane). The standard protocols for RNA synthesis are applied to 5-O-DMT-2'-OMe-3'-O-β-cyanoethyl amidites (0.1 M concentration in dry acetonitrile, < 30 ppm H_2O). The amidites are activated with tetrazole (0.45 M in dry acetonitrile, <30 ppm H_2O). Coupling times are typically up to 15 minutes for 2'-OMe amidites. The phosphonite intermediate is treated with Beaucage reagent to form the phosphorothioate linkage. After each oxidation step, a capping step which places an acetyl group on any remaining uncoupled 5'-OH groups is introduced by treatment with a mixture of two capping reagents (CAP A:acetic anhydride in THF, and CAP B:n-methylimidazole in THF). The cycle is repeated 8 times with various bases to obtain the desired sequence. To the last 3'-OH is coupled a thiol-modifier (thiol modifier C6 S-S, Glen Research 10-1936) that puts a protected disulfide on the oligonucleotide. The DMT is removed with DCA. The support is treated with excess tris-carboxyethyl phosphine (TCEP, Pierce cat. # 20490) and washed to remove the excess TCEP. The resulting sulfhydryl is treated with excess succinimidyl 4-(p-maleimidophenyl)butyrate (SMPB, Pierce cat. # 22315). The support is washed to remove excess SMPB. The resulting NHS ester is reacted with excess histidine hexamer. The resulting oligohistidine oligonucleotide conjugate is then washed to remove excess histidine hexamer. The support is treated at 55 °C in concentrated ammonium hydroxide for 16 hours. The solution is concentrated on a speed vac and the residue taken up in 100 μl of aqueous 0.1 M triethyl ammonium

acetate. This is applied to an HPLC column (C-18, Kromasil, 5 µm, 4.3 mm diameter, 250 mm length) and eluted with an acetonitrile gradient (solvent A: 0.1 M TEAA; solvent B: 0.1 M TEAA and 50% acetonitrile) over 30 minutes at 1 ml/min. flow rate. Fractions of greater than 90% purity are pooled and concentrated.

His 6 Derivatized Cleaver: The oligonucleotide is synthesized in the same fashion as the ACPID cleaver described above, except that a histidine hexamer is used to conjugate to the NHS ester instead of ACPID.

ACPID Derivatized Anchor: The oligonucleotide is synthesized in the same fashion as the Histidine 6 anchor described above except that the ACPID molecule is used to conjugate to the NHS ester instead of histidine hexamer.

Linking ACPID anchor oligonucleotide to His 6 cleaver oligonucleotide: The ACPID anchor oligonucleotide is treated with 10 eq of a 0.1 N solution of $NiSO_4$. The mixture was passed through a G-25 gel filtration spin column to remove the excess nickel. A solution of the His6 cleaver is added to the nickel charged ACPID anchor oligonucleotide. The linkage of the anchor and cleaver through the his6 nickel chelate is confirmed on polyacrylamide gel (19%).

Linking ACPID cleaver oligonucleotide to His 6 anchor oligonucleotide: The ACPID cleaver oligonucleotide is treated with 10 equivalents of a 0.1 N solution of $NiSO_4$. The mixture was passed through a G-25 gel filtration spin column to remove the excess nickel. A solution of the His6 anchor is added to the nickel charged ACPID anchor oligonucleotide. The linkage of the anchor and cleaver through the His6 nickel chelate is confirmed on polyacrylamide gel (19%).

Synthesis of cleavers containing universal bases: Oligonucleotides containing universal bases (5-nitroindole, inosine) were synthesized as described in Example 1 above, substituting the modified monomers for natural bases.

Example 6

(Assay)

Materials: Polymerase chain reaction (PCR) was used to prepare a dsDNA fragment encoding part of secreted alkaline phosphatase (SEAP) using the following

primers:

P3 (SEQ ID NO:3) 5'-cgaaattaaatcgactcactat-3'

P3.1 (SEQ ID NO:4) 3'-gctttaattatgctgagtgatatcccgaagcttagcgcttaagcgggtggtacgacg-acgacgacgacgacgacccggac-5'

P4 (SEQ ID NO:5) 3'-tagggtcaactcctcctcttgg-5'

P5 (SEQ ID NO:6) 3'-tacgacgacgacgacgacgacgacccggactccgatgtcgagagggacccgtagta-gggtcaactcctcctcttgg-5'

These primers are based on the SEAP RNA fragment (1 to 102) having the sequence (SEQ ID NO:7) 5'-.gggcttcgaatcgcgaattcgcccaccatgctgctgctgctgctgc-tgctgggcctgaggctacagctctccctgggcatcatcccagttgaggaggagaacc

The PCR amplification was performed under the manufacturer's (Life Technologies, Cat. No. 10198-018) recommended reaction conditions. Primers P3.1 and P5 were used at 10 nM, while primers P3 and P4 were used at 0.50 µM. The PCR program was 94°C for 5 minutes, 35 cycles of 52°C for 30 seconds, 72°C for 1 minute, 94°C for 45 seconds, then 72°C for 10 minutes.

SEAP dsDNA was then transcribed into ssRNA using a RiboMax™ large Scale RNA kit (Promega, Cat. No. P1300). The SEAP DNA concentration was 30 µg/mL. The transcription reaction was terminated by adding DNase I and incubating at 37°C for 15 minutes. DNA fragments and free nucleotides were removed by precipitation in EtOH/ NaOAc and washing with 70% EtOH. The RNA was resuspended and diluted to approximately 2 µM for use in the RNase H activity assays.

Assay: Test oligonucleotides (20 µM each), SEAP RNA (10 µl of 2 µM solution), and Tris/EDTA buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, "TE", qs to 2 µl) were added to 500 µl thin-wall reaction tubes and incubated for 3 to 5 minutes at 40°C to reach thermal equilibrium. RNase H buffer (10X: 200 mM Tris-HCl, pH 7.4-7.5, 1000 mM KCl, 100 mM $MgCl_2$•$6H_20$, 0.5 mM DTT, 25% w/v sucrose), RNase H (0.4 to 0.6 U, Promega, Cat. No. M4281), and water (qs to 20 µL), were combined to form a cocktail, and incubated for 3 to 5 minutes at 40°C. Then, 8 µl of the cocktail was added to each reaction tube and mixed as quickly as possible to prevent cooling.

Reactions were incubated at 40°C for 30 minutes in an MJ Research PCT-100 temperature controller. Reactions were stopped by adding 20 μl FDE sample buffer (90% v/v formamide, 10% v/v 10X TBE buffer, 0.5% w/v bromophenol blue, 25 mM EDTA) (1X TBE: 89 mM Tris base, 89 mM boric acid, 2 mM EDTA, pH 8.0) to each reaction and heating to 90°C for 3 to 5 minutes.

Detection: Each sample (8 to 10 μl) was run on denaturing 15% polyacrylamide gels at 200 volts for about one hour, or until the dye front had reached the bottom of the gel. Gels were run in electrophoresis cassettes, 8 cm x 8 cm x 1 mm (Novex, Cat. No. NC2010). Gels were poured immediately before use. Briefly, unpolymerized denaturing gel mix (10 ml) was degassed thoroughly under vacuum, combined with 10% ammonium persulphate (35 μl, BioRad) and TEMED (12 μl, BioRad), and poured into each cassette. After polymerization, gels were pre-electrophoresed at 250-300 volts until the current had stabilized at 4 to 5 mA per gel.

Nucleic acid bands in gels were visualized by soaking the gels in a 1:10,000 dilution of CyberGold™ (Molecular Probes, Cat. No. S-11494) in 1X TBE for 5 to 10 minutes, soaking in 1X TBE for an additional 5 - 10 minutes, and irradiating on a short wave UV transilluminator. The results were recorded by photographing the CyberGold™ fluorescence using a CyberGREEN™ filter and a Polaroid MP-4 camera with Polaroid Type 667 3000 ASA black and white film.

Duplex DNA ladders (20 bp and 100 bp, GenSura, San Diego) were used as size standards. Standard ladders were not heated before loading on gels, and were undenatured, running as duplex DNA fragments in both denaturing and non-denaturing gels.

Band Shifts: Gel band shifts were performed with anchor/cleaver pairs in order to demonstrate the high affinity of the oligonucleotides for each other in the absence of the RNA target.

Various anchor and cleaver oligonucleotides were mixed together in 1X RNase H buffer, 15% glycerol, and 6% FDE, and heated to 65°C for 30 seconds. The final concentration of each oligonucleotide was 6.6 μM. After cooling to room temperature, the samples were run directly on a non-denaturing 15% (19:1)

acrylamide gel containing 1 M urea and 1X TBE. Non-denaturing gels for band shift experiments were prepared, run, and visualized the same way as denaturing gels, substituting non-denaturing gel mix for denaturing gel mix.

Complementary cleaver/anchor duplexes containing fluorescently tagged oligonucleotides 1015 and 1016 (1015 = rhodamine, 1016 = fluorescein, and pairs 1000/1016, 1015/1010, 1015/1012, 1015/1013, 1015/1014, and 1015/1016) were demonstrated to hybridize efficiently to each other in the absence of target RNA by gel shift analysis under non-denaturing, stringent conditions. The components are set forth in Table 1. Duplex formation was confirmed by a strong mobility shift in the gel compared to size standards.

TABLE 1: Cleaver and Anchor Molecules

Oligo #	Cleaver or Anchor	SEQ ID NO:	Sequence*
1000	cleaver	8	5'-<u>GCUGGUUGAGUACUC</u>9ggugggcgaauucgc
1010	anchor	9	5'-<u>GCUGGUUGAGUACUC</u>9ggugggcgaauu
1012	anchor	10	5'-<u>GCUGGUUGAGUACUC</u>9ggugggcg
1013	anchor	11	5'-<u>GCUGGUUGAGUACUC</u>9gguggg
1014	anchor	12	5'-<u>GCUGGUUGAGUACUC</u>9ggug
1015	cleaver	13	5'-RGCAGCAGCAT+<u>GAGUACUCAACCAGC</u>
1016	anchor	14	5'-F<u>GCUGGUUGAGUACUC</u>+ggugggcgaa

where "ACGT" indicates PS DNA, "<u>ACGT</u>" indicates 2'-OMe RNA, "acgt" indicates 2'-OMe PS RNA, and "9" indicates Glen Research linker #9.

TABLE 2: duplex formation in the absence of target RNA

Cleaver/Anchor	Relative Mobility, denaturing conditions	Relative Mobility of Complex, non-denaturing conditions
1015	25	25
1015/1010	25/27	44
1015/1012	25/23	39
1015/1013	25/21	37
1015/1014	25/19	35
1016	25	25
1016/1000	25/27	41
1016/1015	25/25	39

Gels were photographed before and after CyberGold staining to visualize the fluorescently labeled oligonucleotides (1015 and 1016) alone, and in complexes with unlabled oligonucleotides. Both photographs were identical except for the DNA standard ladders revealed by the CyberGold fluorescence.

Melting Point Determination: The melting point of the 15 base 2'-O-methyl RNA duplex stem used to bring the cleavers and anchors together was determined by UV spectroscopy. A Carey 3E (Varian) spectrophotometer with a thermal controller was used to monitor the absorbance of anchor/cleaver pairs 1000/1010 and 1000/1013 in MP buffer (150 mM NaCl, 10 mM Na_2HPO_4, 0.1 mM EDTA, pH 7.4).

An increase in absorbance of 0.1 AU at 260 nm at 75°C indicated that the melting temperature of the duplex stem was 75°C. A melting temperature this high implies that the complex is very long lived, with an off rate of several days.

The results demonstrated that the 15 base 2'-OMe RNA stem binding GeneLead anchors and cleavers together into stable and functional antisense molecules, and that association occurred in the absence of target RNA, and in the presence of 1M urea. With a melting temperature of 75°C, the duplex stem is capable

of binding GeneLead library member molecules together in solution, during cell transfection, and during the exertion of an antisense effect on specific target RNA molecules.

Example 7

(Activity)

Cleaver and anchor oligonucleotides were synthesized in decreasing lengths and tested for RNase H activation on SEAP. The following oligonucleotides were prepared:

Oligo #	SEQ ID NO:	Sequence
1000†	15	5'-CAGCAGCAGCAT9GAGUACUCAACCAGC
1006†	16	5'-GCAGCAGCAT9GAGUACUCAACCAGC
1007†	17	5'-AGCAGCAT9GAGUACUCAACCAGC
1008†	18	5'-CAGCAT9GAGUACUCAACCAGC
1009†	19	5'-GCAT9GAGUACUCAACCAGC
1034†	20	5'-CAGCAT-GAGUACUCAACCAGC
1001‡	21	5'-GCUGGUUGAGUACUC9ggugggcgaauucgc
1010‡	22	5'-GCUGGUUGAGUACUC9ggugggcgaauu
1011‡	23	5'-GCUGGUUGAGUACUC9ggugggcgaa
1012‡	24	5'-GCUGGUUGAGUACUC9ggugggcg
1013‡	25	5'-GCUGGUUGAGUACUC9ggugggg
1014‡	26	5'-GCUGGUUGAGUACUC9ggug
1035‡	27	5'-GCUGGUUGAGUACUC-ggugggcg
1045‡	28	5'-GCUGGUUGAGUACUC9ggugggcgaauucgc1

where "ACGT" indicates phosphorothioate deoxyribonucleic acids, "ACGT" indicates 2'-O-methyl ribonucleic acid, "9" indicates Glen Research linker #9, "1" indicates Glen Research propyl linker on CPG, † indicates a cleaver oligo, and ‡ indicates an anchor oligo.

Table 3: RNase H activation - Cleaver Size

Cleaver #	Cleaver binding length	Anchor #	Anchor binding length	RNA cleavage w/o anchor	RNA cleavage with anchor
1000	12	1001	15	+++++	++++++
1006	10	1001	15	+++	++++
1007	8	1001	15	++	+++
1008	6	1001	15	-	+
1009	4	1001	15	-	-

All cleaver oligonucleotides resulted in more efficient cleavage when combined with the 1001 anchor. Cleaver 1008 was active only when combined with an anchor. Cleaver 1009 was inactive, in the presence and absence of the 1001 anchor molecule. The 1000/1001 complex is illustrated in FIG. 3.

Table 4: RNase H activation - Anchor Size

Cleaver #	Cleaver binding length	Anchor #	Anchor binding length	RNA cleavage w/o anchor	RNA cleavage with anchor
1007	8	-	NA	++	NA
1007	8	1010	12	NA	+++
1007	8	1011	10	NA	+++
1007	8	1012	8	NA	+++
1007	8	1013	6	NA	++++
1007	8	1014	4	NA	+++

This data demonstrates that a cleaver of length 8 is capable of stimulating cleavage regardless of the length of any accompanying anchor, but that cleavage is maximized by an anchor of length 6.

Cleavers (six bases) and anchors (eight bases) were prepared with and without a 9-atom linker, and tested for RNase H activity.

Oligo #	SEQ ID NO:	Sequence
1019	29	5'-AGCABBBB9<u>GAGUACUCAACCAGC</u>
1020	30	5'-AGCAGCBB9<u>GAGUACUCAACCAGC</u>
1021	31	5'-BBBBGCAT9<u>GAGUACUCAACCAGC</u>
1022	32	5'-KKPKKPKP9<u>GAGUACUCAACCAGC</u>
1023	33	5'-KKPKGCAT9<u>GAGUACUCAACCAGC</u>

where "ACGTBK" indicate PS DNA, "<u>ACGT</u>" indicates 2-OMe RNA, and "9" indicates Glen Research linker #9. The results are set forth in Table 5 below:

Table 5: RNase H activity of oligonucleotides having linkers

Cleaver #	Linker (y/n)	Anchor #	Linker (y/n)	RNA cleavage w/o anchor	RNA cleavage with anchor
1008	Y	none	NA	-	NA
1008	Y	1012	Y	NA	++++
1008	Y	1035	N	NA	++
1034	N	none	NA	-	NA
1034	N	1012	Y	NA	+++
1034	N	1035	N	NA	+

The results demonstrated increased activity when flexible linkers were used. The greatest activity was obtained when both cleaver and anchor contained a linker. In complexes having only one linker, the linker had the greatest effect when present in the anchor portion.

Cleavers and anchors were prepared incorporating modified bases.

"Universal bases" ("B") do not significantly hydrogen bond to any natural base, but are tolerated in the duplex structure. "Degenerate bases" are those that hydrogen bond or fit in a duplex with either purines or pyrimidines ("K" and "P", respectively), but not both. By substituting a number of universal or degenerate bases for the natural bases in cleavers and/or anchors, one can prepare oligos having the ability to bind a greater number of target mRNA sequences.

Table 6: Activity with Cleaver length of 6 or 8

Anchor #	Anchor binding length	Cleaver #	Cleaver binding length	RNA cleavage, w/o anchor	RNA cleavage, with anchor
none	NA	1007	8	++	NA
1010	12	1007	8	NA	+++
1013	6	1007	8	NA	+++++
1014	4	1007	8	NA	+++
none	NA	1008	6	-	NA
1010	12	1008	6	NA	+
1013	6	1008	6	NA	++
1014	4	1008	6	NA	-

The results from this experiment demonstrated that cleavers having a length of 8 bases were more effective in obtaining cleavage than 6 base cleavers, and that anchors having a length of 6 bases were more effective than 4 base or 12 base anchors.

Table 7: Activity with non-natural bases

Cleaver #	N/B/P-K	Anchor #	RNA cleavage activity	Library size
1007	8/0/0	none	++	65,536
1007	8/0/0	1013	++++	65,536
1019	4/4/0	none	-	256
1019	4/4/0	1013	+	256
1020	6/2/0	none	++	4096
1020	6/2/0	1013	++++	4096
1021	4/4/0	none	-	256
1021	4/4/0	1013	-	256
1022	0/0/8	none	-	256
1022	0/0/8	1013	-	256
1023	4/0/4	none	-	1024
1023	4/0/4	1013	-	1024
1052	6*/0/6	1012	++	262,144

*Propynyl pyrimidine bases and diaminopurine were used.

In Table 7 above, all cleavers have 8 bases, and the anchor has 6 bases. "N" indicates the number of natural bases, "B" indicates the number of universal bases, and "P-K" indicates the number of degenerate purine/pyrimidine bases. The "library size" is the number of molecules that would constitute every possible oligo of the size and composition set forth.

The results indicate that cleaver 1020, having 2 universal bases, bound as effectively as cleaver 1007 (having only natural bases), in the presence or absence of anchor oligos. Note that the size of the corresponding libraries is reduced by a factor of 16 by incorporating two universal bases.

Cleaver oligonucleotides including the universal base "B" having the

sequences set forth in Table 8 were synthesized and tested for RNase H activity. The results are set forth in Table 9:

Table 8: Oligos

Cleaver #	SEQ ID NO:	Sequence*
1033	34	5'CAGCAT9ggugggcg1
1025	35	5'GCAT9ggugggcgaauu1
1026	36	5'AGCABBBB9ggugggcgaauu1
1027	37	5'GCBB9ggugggcgaauu1

* C, G, A, T = phosphorothioate DNA: c, g, u, a = 2'-O-Me phosphorothioate RNA; "9" = Glen Research Liner #9, "1" = Glen Research propyl linker

Table 9: Cleaver oligonucleotides including B

Cleaver #	2'O-Me/RNase H/Universal bases	Anchor #	RNA cleavage
1033	8/6/0	None	++
1025	12/4/0	None	-
1026	12/8/4	None	+++
1027	12/2/2	None	-
1019	6/8/4	1013	+
1020	6/8/2	1013	++++

The presence of a flexible linker between the RNase H recognition region and the 2'-OMe RNA binding region of 1033 does not eliminate the antisense activity. This linker appears to be tolerated and serves as a model for other ways to join a target-cleaving region and target region to form a single, active antisense molecule. Other ways of joining cleavers and anchors, two short cleavers, and other library-based oligonucleotide structures will (for example chelation and post-synthetic covalent

interaction) may be substituted.

The RNase H activity of 1033 (in comparison to 1025) clearly demonstrates a length dependence on RNase H recognition. Even thought the overall length of the molecule is longer for 1025, it is less active than 1033. The key difference appears to be in the length of the short, all-phosphorothioate (PS) region. A 4-base PS region at the end of the molecule appears to be too short for efficient cleavage. Thus, 1027 is most likely inactive due to the fact that the RNase H substrate PS region is only 4 bases long, and not due to the 2 universal bases.

A comparison of the RNase H activity of 1033 (a single, linear antisense molecule) with the 1020/1013 pair demonstrates the dependence on the length of the RNase H substrate region. The 1020/1013 pair, containing two universal bases and a duplex stem holding the structure together, is significantly more active than 1033, which contains no universal bases, no stem structure, and has exactly the same footprint length on the SEAP target mRNA.

The two universal bases contained in 1020 appear to lengthen the all-PS RNase H recognition region just enough, to 8 total bases, that the target RNA is cleaved much more efficiently than RNA hybridized to the 6 base substrate region of 1033. The critical step of including universal bases to avoid increasing the numerical complexity of an antisense library is graphically demonstrated by these results. The 6 base PS region of 1033 contributes a factor of 4,096 to any library based on this linear, linker-containing oligonucleotide. Adding two natural bases to the PS region to improve its RNase H substrate activity would increase the factor by 4^2, to a total of 65,536. The fact that the 1020/1013 pair, containing two universal bases and a bulky 2'-OMe duplex stem coupler, is more active than a linear molecule is surprising.

Example 8

(Intracellular Activity)

Protein Kinase C Alpha (PKCα) was chosen as the gene target to demonstrate activity inside human cells. PKCα is a normal human gene that is overexpressed in a majority of human cancer types, and is one of the most highly

publicized of all antisense target genes.

The oligonucleotides prepared for this example are listed in Table 10.

Table 10: Oligonucleotides

Oligo #	SEQ ID NO:	Cleaver/ Anchor	Sequence*
1040	38	cleaver	5'GTTCTCGCTGGT9GAGUACUCAACCAGC1
1041	39	anchor	5'GCUGGUUGAGUACUC9gaguuuca
1042	40	control cleaver	5'TGTGTTACCATC9GAGUACUCAACCAGC1
1043	41	control anchor	5'GCUGGUUGAGUACUC9gguugcgu
1058	42	ISIS3521 antisense	5'GTTCTCGCTGGTGAGTTTCA
1059	43	ISIS4189 control	5'GGTTTTACCATCGGTTCTGG
1061	44	BCL2 4 mismatch control	5'TCTACCCGCGTCCGGCAT

* C, G, A, T = phosphorothioate DNA: c, g, u, a = 2'-O-Me phosphorothioate RNA; C, G, A, T = 2'-OMe RNA; "9" = Glen Research Linker #9, "1" = Glen Research propyl linker

Oligo 1040 (a 12-mer, RNase H-substrate cleaver) hybridized to 1041 (an 8-mer, non-RNase H-substrate anchor) to create an active antisense construction against PKCα. Oligos 1042 and 1043 were a control clever and anchor, respectively, that hybridized together to form a construct that does not match a known gene, but has the same base composition as 1059 (ISIS4189), a control all-phosphorothioate

oligonucleotide 20-mer. Oligo 1058 (ISIS3551) was a conventional 20mer all-phosphorothioate antisense oligonucleotide that has been well established to act via an antisense mechanism to down regulate the expression of PKCα. Oligo 1061 was a conventional all-phosphorothioate 18mer, 4 base mismatch control to the BCL2 gene.

A human bladder carcinoma line (T-24, ATCC HTB-4), a cell line known to overexpress PKCα, was used in the experiments. T-24 was maintained in culture using standard methods: 37°C, 5% CO_2, in 75 cm^2 flasks (Falcon, 3084) in McCoy's 5A medium (Mediatech, # 10-050-CV) with 10% serum (Gemini Bio-Products, #100-107) and penicillin-streptomycin (50 IU/ml, 50 μg/ml, Mediatech #30-001-LI). For antisense experiments, T-24 cells were plated into 12-well plates (Falcon, #3043) at 75,000 cells/well and allowed to adhere and recover overnight before transfection.

Oligonucleotides were transfected into T-24 cells with a cationic lipid-containing cytofection agent (LipofectACE™, GibcoBRL, #18301-010), which provides efficient nuclear delivery of fluorescently labeled oligonucleotides of the invention in T-24.

Oligonucleotides of the invention and conventional all-phosphorothioate oligonucleotides were diluted into 1.5 mL of reduced serum medium Opti-MEM® I (Gibco-BRL, #11058-021) to a concentration of 400 nM each. The oligonucleotide-containing solutions were then mixed with an equal volume of Opti-MEM I containing LipofectACE sufficient to give a final lipid to oligonucleotide ratio of 5 to 1 by weight. The final concentration of oligonucleotide was 200 nM. The oligonucleotide/lipid complexes were incubated at room temperature for 20 minutes before adding to tissue culture cells.

Cells were washed once in phosphate buffered saline (PBS, Mediatech, #21-030-LV) to rinse away serum-containing medium, and then transfection mix (1 ml) was placed in each well of a 12-well plate. All transfections were performed in triplicate. The cells were allowed to take up oligonucleotide/lipid complexes for 22 hours prior to harvesting the total cellular RNA. Mock transfections consisted of cells treated with Opti-MEM I only.

<u>Total Cytoplasmic RNA Isolation</u>: After 22 hours of antisense treatment,

total RNA was harvested from the cells. The cells were released from the plates by trypsinizing (Trypsin/ EDTA, Mediatech #25-052-LI) according to standard methods. The triplicate groups of cells were pooled and total cytoplasmic RNA was isolated using an RNeasy Kit (QIAGEN, #74104) according to manufacturer's protocols. The RNA was DNase I treated and UV quantitated according to standard methods.

Polymerase Chain Reactions to Detect PKCα RNA: Reverse Transcriptase/ Polymerase Chain Reactions (RT-PCR) were performed with the methods and materials from a SuperScript One-Step RT-PCR Kit from GibcoBRL (Cat. No. 10928-026). The RT-PCR reactions to detect PKCα were performed in two independent runs, with PKCα-specific primers from Oxford Biomedical Research (#EZ-60A and EZ-60B) and 100 ng of input total RNA.

Control Multiplex RT-PCRs (MP RT-PCRs) were performed to confirm equal quantities of input RNA into each PKCα RT-PCR. The primers, reagents, and protocol were from Maxim Biotech (#APO-M052-G). The control MP RT-PCRs amplified BAX and LICE genes equally in all samples, confirming that equal amounts of intact RNA were added to the PKCα RT-PCRs.

All RT-PCR reactions were performed according to the following program on a PTC-100 thermocycler (MJResearch): Step 1, 50°C for 35 minutes; Step 2, 94°C for 2 minutes; Step 3, 55°C for 30 seconds; Step 4, 72°C for 1 minute; Step 5, 94°C for 30 seconds; Step 6, Go to Step 3, 33 more times; Step 7, 72°C for 10 minutes; Step 8, End. All RT-PCR products were separated on a 4% Super Resolution Agarose TBE gel (Apex, #20-105) and stained with CyberGold (Molecular Probes, #S-11494), according to the manufacturer's instructions. Gels were photographed on Polaroid Type 667 film. The results are set forth in Table 11.

Table 11: Intracellular RNase H activity

Treatment	1st oligo	2nd oligo	PKCα	BAX	LICE
Mock treatment	-	-	++++	++++	+++
Cleaver alone	1040	-	++++	++++	+++
Anchor alone	-	1041	++++	++++	+++

Treatment	1st oligo	2nd oligo	PKCα	BAX	LICE
Anchor + Cleaver	1040	1041	+	++++	+++
Control	1042	1043	++++	++++	+++
Conventional antisense	1058	-	+	++++	+++
Conventional control	1059	-	++++	++++	+++
Conventional control	1061	-	++++	++++	+++

The oligonucleotide constructs of the invention proved to be as active as conventional 20mer phosphorothioate oligonucleotides, as demonstrated by the anchor+cleaver vs conventional antisense above. Note that neither cleaver (1040) nor anchor (1041) demonstrated any activity when administered alone, but demonstrated full activity when assembled. The control GeneLead construct (1042+1043) showed no non-specific activity against PKCα, BAX or LICE, nor did any of the other control oligonucleotides.

The results demonstrate that GeneLead constructs are as active as conventional antisense molecules. Further, the GeneLead constructs can be assembled from a standing, pre-synthesized library of components, which is not feasible with conventional antisense molecules.

Example 9

(Activity Against the Human Bcl2 Gene in Tissue Culture Cells)

B-cell Lymphoma-Associated Gene 2 (BCL2) was chosen to demonstrate GeneLead™ activity inside human cells. BCL2 is another "normal" human gene that is over expressed in a majority of human cancer types. The BCL2 protein is one of a large family of proteins that regulate cell death. BCL2 over expression is known to cause cells to be chemotherapy and radiotherapy resistant.

Oligomers: The following BCL2-targeted antisense molecules were synthesized:

1060 BCL2 18-base antisense 5'TCTCCCAGCGTGCGCCAT (SEQ ID NO:45)

1061 BCL2 4 mismatch control 5'TCTACCCGCGTCCGGCAT (SEQ ID NO:46)

1062 BCL2 GeneLead Cleaver 5'TCTCCCAGCGTG9<u>GAGUACUCAACCAGC</u>1 (SEQ ID NO:47)

1063 BCL2 GeneLead Cleaver 5'<u>***TCTCCC***</u>AGCGBB9<u>GAGUACUCAACCAGC</u>1 (SEQ ID NO:48)

1066 BCL2 GeneLead Anchor 5'<u>GCUGGUUGAGUACUC</u>9cgccat1 (SEQ ID NO:49)

where NNNN = phosphorothioate deoxyribonucleic acid (PS DNA), <u>NNNN</u> = 2'-O-methyl ribonucleic acid (2'-OMe RNA), nnnn = 2'-O-Methyl phosphorothioate ribonucleic acid (2'-OMe PS RNA), and <u>*NNNN*</u> = C-5 Propynyl-modified phosphorothioate deoxyribonucleic acid (Propynyl), 9 = Glen Research linker #9, 1 = Glen Research propyl linker on CPG (Cat. No. **), F = Molecular Probes Fluorescein (Cat. No. F-1907), and R = Molecular Probes Rhodamine (Cat. No. X-491).

1062 (a 12-mer, RNase H-substrate cleaver) and 1063 (a 12-mer, RNase H-substrate cleaver with a 6-base C-5 propynyl-modified "tack" at the 5' end of the RNase H-substrate region) both hybridized to 1066 (a 6-mer, non-RNase H-substrate anchor) to create active GeneLead antisense constructions against BCL2.

1060 (based on a published oligonucleotide known clinically as G3139) is a conventional 18-mer all-phosphorothioate antisense oligonucleotide. 1060 hybridizes to the BCL2 pre-mRNA across the first 6 codons of the open reading frame.

1061 is a conventional all-phosphorothioate 18-mer, 4 base mismatch control to the BCL2 gene.

<u>Tissue Culture</u>: The cell line that was used for this demonstration was T-24 (American Type Culture Collection #HTB-4), a human bladder carcinoma line known to over express BCL2.

T-24 was maintained in culture using standard methods at 37°C, 5% CO_2, in 75-cm^2 flasks (Falcon, Cat. No. 3084) in McCoy's 5A medium (Mediatech, Cat. No. 10-050-CV) with 10% serum (Gemini Bio-Products, Cat. No. 100-107) and penicillin-streptomycin (50 IU/mL, 50 mcg/mL, Mediatech, Cat. No. 30-001-LI).

For antisense experiments T-24 were plated into 12-well plates (Falcon,

Cat. No. 3043) at 75,000 cells/well and allowed to adhere and recover overnight before oligonucleotide transfections began.

Transfection of Oligonucleotides into T-24 cells: Oligonucleotides were transfected into T-24 cells with a cationic lipid-containing cytofectin agent LipofectACE™ (GibcoBRL, Cat. No. 18301-010). LipofectACE has been shown to give efficient nuclear delivery of fluorescently labeled GeneLead constructions in T-24.

GeneLead and conventional all-phosphorothioate oligonucleotides were diluted into 1.5 mL of reduced serum medium Opti-MEM© I (GibcoBRL, Cat. No. 11058-021) to a concentration of 400 nM each. The oligonucleotide-containing solutions were then mixed with an equal volume of Opti-MEM I containing LipofectACE sufficient to give a final lipid to oligonucleotide ratio of 5 to 1 by weight.

The final concentration of oligonucleotide was 200 nM. The oligonucleotide/lipid complexes were incubated at room temperature for 20 minutes before adding to tissue culture cells.

Cells were washed once in phosphate buffered saline (PBS, Mediatech Cat. No. 21-030-LV) to rinse away serum-containing medium and then one mL of transfection mix was placed into each well of a 12-well plate. All transfections were performed in triplicate.

The cells were allowed to take up oligonucleotide/lipid complexes for 24 hours prior to harvesting of total cellular RNA. Mock transfections consisted of cells treated with Opti-MEM I only.

Total Cytoplasmic RNA Isolation: After 22 hours of antisense treatment, total RNA was harvested from the cells. The cells were released from the plates by trypsinizing (Trypsin/EDTA, Mediatech Cat. No. 25-052-LI) according to standard methods. The triplicate groups of cells were pooled and total cytoplasmic RNA was isolated according to the RNeasy Protocol and spin columns from an RNeasy Kit (QIAGEN, Cat. No. 74104).

The RNA was DNase I treated and UV quantitated according to standard

methods.

Polymerase Chain Reactions to Detect BCL2 RNA: Reverse Transcriptase/ Polymerase Chain Reactions (RT-PCR) were performed with the methods and materials from a SuperScript One-Step RT-PCR Kit from GibcoBRL (Cat. No. 10928-026). The RT-PCR reactions to detect BCL2 were performed with BCL2-specific primers from the literature: upstream 5'ggtgccacctgtggtccacctg and downstream 5' cttcacttgtggcccagatagg (both primers were normal DNA) and 1 μg of input total RNA. Control RT-PCR reactions against β-actin were also performed with primers from the literature: upstream 5' gagctgcgtgtggctcccgagg and downstream 5' cgcaggatggcatgggggcatacccc (both primers were normal DNA) and 0.1 g of input total RNA.

All BCL2 and β-actin RT-PCR reactions were performed according to the following program on a PTC-100 thermocycler (MJResearch): Step 1, 50°C for 35 minutes; Step 2, 94°C for 2 minutes; Step 3, 60°C for 30 seconds; Step 4, 72°C for 1 minute; Step 5, 94°C for 30 seconds; Step 6, Go to Step 3, 35 more times; Step 7, 72°C for 10 minutes; Step 8, End.

All RT-PCR products were separated on a 4% Super Resolution Agarose TBE gel (Apex, Cat. No. 20-105) and stained with SyberGold (Molecular Probes, Cat. No. S-11494), according to the manufacture's instructions. Gels were photographed on Polaroid Type 667 film.

Table 12: Reduced Target Gene Expression (BCL2) Confirms that GeneLead Constructions With Universal Bases Are Active and Specific in Cells

Lane	Treatment	Cleaver Oligo	Anchor Oligo	All-PS Oligo	BCL2 mRNA level	β-actin mRNA level
1	Mock	-	-	-	++++	++++
2	Conventional antisense		-	1060	+	++++

Lane	Treatment	Cleaver Oligo	Anchor Oligo	All-PS Oligo	BCL2 mRNA level	β-actin mRNA level
3	Conventional control	-	-	1061	++++	++++
4	Cleaver alone	1062	-		++++	++++
5	GeneLead assembled	1062	1066	-	+	++++
6	Cleaver alone	1063	-	-	+++	++++
7	GeneLead assembled	1063	1066	-	+	++++
8	Anchor alone	-	1066	-	++++	++++

Results

The GeneLead anti-BCL2 constructions dropped BCL2 RNA levels significantly compared to control treatments. Compare lanes 5 (oligos 1062+1066) and 7 (1063+1066) to lanes 1 (mock treatment) and 3 (conventional antisense control).

None of the oligonucleotides and GeneLead constructions showed any activity against the control gene β-actin.

This is significant because it clearly demonstrates GeneLead activity with: (a) only a 6 base anchor (1066, lanes 5 and 7), (b) two nitroindole universal bases, "B", replacing natural bases in the cleaver sequence (1063 alone, and 1063+1066, lanes 6 and 7), and (c) that GeneLead activity is general and could be easily observed against another human target genes.

The experimental result that an anchor as short a 6 bases long combined with a cleaver containing nitroindole as a universal base (1063+1066) could form a GeneLead construct with effective antisense activity inside cells clearly confirmed the validity of our cell-free work with SEAP-targeted GeneLead oligonucleotides.

The principles of separating oligonucleotides into two or more functional units and incorporating universal bases in order to numerically simplify combinatorial libraries of antisense oligonucleotides has been reduced to practice in living human cells.

These concepts can easily be applied to improve current uses of oligonucleotides in diagnostics, ribozyme applications, and immuno-stimulation (CpG oligonucleotides).

REFERENCES

Ramon Eritja et al., "Synthesis and properties of defined DNA oligomers containing base mispairs involving 2-aminopurine" Nuc Acids Res (1986) 14(14):5869-85
Stefan Pitsch et al., "Why Pentose-and Not Hexose-Nucleic Acids?" Helv Chimica Acta (1993) 76:2161-83
P. Kong Thoo Lin et al., "Synthesis and duplex stability of oligonucleotides containing cytosine-thymine analogues" Nuc Acids Res (1989) 17(24):10373-83
Daniel M. Brown et al., "Synthesis and duplex stability of oligonucleotides containing adenine-guanine analogues" Carbohydrate Res (1991) 216:129-39
D. Loakes et al., "5-Nitroindole as an universal base analogue" Nuc Acids Res (1994) 22(20):4039-43
Novabiochem Catalog and Peptide Synthesis Handbook, 1997-1998, pp. S1-S88
E. Hochuli et al., "New Metal Chelate Adsorbent Selective for Proteins and Peptides Containing Neighboring Histidine Residues" J.Chromatog (1987) 411:177-84
Eric E. Swayze et al. "The Synthesis of N,N'-O-Trisubstituted Hydroxylamines via a Mild Reductive Alkylation Procedure: An Improved Synthesis of the MMI Backbone" Synlett (1997) 859-861.
Francois Morvan et al. "Oligonucleotide Mimics for Antisense Therapeutics:Solution Phase and Automated Solid-Support Synthesis of MMI Linked Oligomers" J Am Chem Soc (1996) 118:255-56

Michel Perbost et al. "Synthesis of 5'-O-Amino-2'deoxypyrimidine and Purine Nucleosides: Building Blocks for Antisense Oligonucleotides" J. Org. Chem. (1995) 60:5150-56
Antivirals Inc., "General Properties of Morpholino Oligomers",Technical Bul#1, 1998
Kim L. Dueholm et al. "Synthesis of Peptide Nucleic Acid Monomers Containing the Four Natural Nucleobases: Thymine, Cytosine, Adenine, and Guanine and their Oligomerization" J. Org. Chem. (1994) 59:5767-73
Jeffrey S. Nelson et al. "N3'-P5' Oligodeoxyribonucleotide Phosphoramidates: A New Method of Synthesis Based on a Phosphoramidite Amine-Exchange Reaction" J. Org. Chem. (1997) 62:7278-87
Jer-Kang Chen et al. "Synthesis of Oligodeoxyribonucleotide N3'-P5' phosphoramidates" Nuc Acids Res (1995) 23(14):2661-68
Sarah N. McCurdy et al. "An Improved Method for the Synthesis of N3'-P5' Phosphoramidate Oligonucleotides" Tetrahedron Lett (1997) 38(2):207-10

What is claimed:

1. A composition comprising:
a first oligonucleotide analog, comprising a first binding domain capable of binding a target polynucleotide, and a first coupling moiety capable of binding to a second coupling moiety;
a second oligonucleotide analog, comprising a second binding domain capable of binding a target polynucleotide, and a second coupling moiety capable of binding to said first coupling moiety;
wherein said first and second coupling moieties are capable of coupling in the absence of a target polynucleotide.

2. The composition of claim 1, wherein said first and second binding domains each independently comprise from about 3 to about 24 bases.

3. The composition of claim 1, wherein said first and second binding domains each independently comprise a backbone and a plurality of bases.

4. The composition of claim 3, wherein said backbone is selected from the group consisting of ribonucleic acid, deoxyribonucleic acid, DNA phosphorothioate, RNA phosphorothioate, 2'-O-hydrocarbyl ribonucleic acid, 2;-O-hydrocarbyl DNA, 2'-O-hydrocarbyl RNA phosphorothioate, 2'-O-hydrocarbyl DNA phosphorothioate, 2'-F-phosphorothioate, 2'-F-phosphodiester, 2'-methoxyethyl phosphorothioate, 2-methoxyethyl phosphodiester, deoxy MMI, 2'-O-hydrocarby MMI, deoxy-methylphosphonate, 2'-O-hydrocarbyl methylphosphonate, morpholino, 4'-thio DNA, 4'-thio RNA, peptide nucleic acid, 3'-amidate, deoxy 3'-amidate, and 2'-O-hydrocarbyl 3'-amidate.

5. The composition of claim 1, wherein said first and second binding

domains each independently comprise a backbone and a plurality of nucleotide bases, wherein said nucleotide bases are selected from the group consisting of natural nucleotide bases, unnatural nucleotide bases, universal nucleotide bases, and degenerate nucleotide bases.

6. The composition of claim 1, wherein at least one of said binding domains is capable of activating or recruiting a nuclease.

7. The composition of claim 6, wherein said nuclease is RNase H.

8. The composition of claim 6, wherein said nuclease is selected from the group consisting of RNase P and RNase L

9. The composition of claim 1, wherein said first oligonucleotide analog and said second oligonucleotide analog when coupled form a ribozyme.

10. The composition of claim 1, wherein said first binding domain and said first coupling moiety are joined together by a flexible linker.

11. The composition of claim 1, wherein said second binding domain and said second coupling moiety are joined together by a flexible linker.

12. The composition of claim 11, wherein said first binding domain and said first coupling moiety are joined together by a flexible linker.

13. The composition of claim 11, wherein said flexible linker comprises polyethylene glycol.

14. The composition of claim 12, wherein said flexible linker comprises polyethylene glycol.

15. The composition of claim 13, wherein said flexible linker comprises polyethylene glycol having from 1 to about 10 ethylene glycol monomers.

16. The composition of claim 14, wherein said flexible linker comprises polyethylene glycol having from 1 to about 10 ethylene glycol monomers.

17. The composition of claim 1, wherein said first and second coupling moieties are selected from the group consisting of oligonucleotide duplexes, oligonucleotide analog duplexes, and protein-ligand pairs.

18. The composition of claim 1, wherein said first and second coupling moieties are selected to participate in a non-covalent binding interaction.

19. The composition of claim 18, wherein said first and second coupling moieties comprise a histidine oligomer and a metal ion-binding moiety.

20. The composition of claim 18, wherein said first and second coupling moieties comprise a phenanthroline moiety and a Zn complex.

21. The composition of claim 1, further comprising a third oligonucleotide analog, comprising a third binding domain capable of binding a target polynucleotide, and a third coupling moiety capable of binding a fourth coupling moiety;
wherein said first oligonucleotide analog or said second oligonucleotide analog further comprises a fourth coupling moiety.

22. The composition of claim 21, further comprising a fourth oligonucleotide analog, comprising a fourth binding domain capable of binding a target polynucleotide, and a fifth coupling moiety capable of binding a sixth coupling moiety;
wherein said first or second or third oligonucleotide analog further comprises a sixth coupling moiety.

23. A compound of formula 1:

$$R_1\text{-}L_1\text{-}X\text{-}A\text{-}Y\text{-}L_2\text{-}R_2,$$

wherein
R_1 is an oligonucleotide, or an oligonucleotide analog, capable of binding to RNA;
R_2 is an oligonucleotide, or an oligonucleotide analog, capable of binding to RNA;
L_1 and L_2 are each independently a linking moiety or a bond;
X and Y are each independently a coupling moiety; and
A comprises a link selected from the group consisting of a covalent bond, a metal ion, and a non-covalent bond
wherein said compound is capable of activating a nuclease or catalyzing cleavage when bound to a target polynucleotide.

24. The compound of claim 23, wherein R_2 is capable of activating RNase H in vivo.

25. The compound of claim 23, wherein said compound comprises a ribozyme.

26. The compound of claim 23, wherein X and Y comprise a pair of complementary oligonucleotides or complementary oligonucleotide analogs.

27. The compound of claim 26, wherein X and Y are coupled to R_1 and R_2 in a polarity opposite to the polarity of R_1 and R_2.

28. The compound of claim 23, wherein A comprises a metal ion, and X and Y comprise ligands capable of simultaneously binding said metal ion.

29. The compound of claim 28, wherein at least one of X and Y comprises a histidine oligomer.

30. The compound of claim 23, wherein at least one of R_1 and R_2 comprises a plurality of oligonucleotide bases selected from the group consisting of universal bases and degenerate bases.

31. The compound of claim 30, wherein R_2 comprises from about 1 to about 20 oligonucleotide bases selected from the group consisting of universal bases and degenerate bases.

32. The compound of claim 23, wherein L_2 comprises a linking moiety.

33. The compound of claim 32, wherein said linking moiety comprises polyethylene glycol.

34. The compound of claim 32, wherein L_1 comprises a linking moiety.

35. The compound of claim 34, wherein said linking moiety comprises polyethylene glycol.

36. A method for cleaving a target RNA molecule, comprising:
providing a target RNA molecule;
contacting the target RNA molecule with a first oligonucleotide analog, comprising a first binding domain capable of binding a first region of a target polynucleotide, and a first coupling moiety capable of binding to a second coupling moiety, and a second oligonucleotide analog, comprising a second binding domain capable of binding a second region of said target polynucleotide, and a second coupling moiety capable of binding to said first coupling moiety, wherein said first and second binding domains are capable of binding simultaneously to said target RNA molecule; and
incubating said target RNA molecule, first analog and second analog in the presence of an RNase capable of cleaving the RNA target.

37. The method of claim 36, wherein said first and second binding domains each independently comprise from about 3 to about 24 bases.

38. The method of claim 36, wherein said first region and second region are non-overlapping.

39. The method of claim 36, wherein said second binding domain and said second coupling moiety are joined together by a flexible linker.

40. The method of claim 39, wherein said first binding domain and said first coupling moiety are joined together by a flexible linker.

41. The method of claim 36, wherein said incubating is intracellular.

42. A method for cleaving a target polynucleotide, comprising:
providing a target RNA molecule;
contacting the target RNA molecule with a first oligonucleotide analog, comprising a first binding domain capable of binding a first region of a target polynucleotide, and a first coupling moiety capable of binding to a second coupling moiety, and a second oligonucleotide analog, comprising a second binding domain capable of binding a second region of said target polynucleotide, and a second coupling moiety capable of binding to said first coupling moiety, wherein said first and second binding domains are capable of binding simultaneously to said target RNA molecule, wherein said first and second target polynucleotide regions are separated from each other by a nucleotide, wherein said first and second oligonucleotide analogs together form a ribozyme; and
incubating said target RNA molecule, first analog and second analog together.

43. The method of claim 42, wherein said first oligonucleotide analog comprises a compound of the formula 5'-GGNNNNNCUGAUGA-X, and said second oligonucleotide analog comprises a compound of the formula 5'-Y-GAANNNNN, where X and Y are coupling moieties capable of coupling with each other, and NNNNN are oligonucleotide bases or oligonucleotide base analogs capable of hybridizing to said first and second target polynucleotide regions.

44. An antisense library, comprising:
a set of first oligonucleotide analogs, each first analog comprising a first coupling moiety and a first binding domain, said first binding domain comprising a first backbone and a plurality of first bases capable of base-pairing with a target nucleic acid; and
a set of second oligonucleotide analogs, each second analog comprising a

second coupling moiety capable of coupling specifically to said first coupling moiety, and a second binding domain, said second binding domain comprising a second backbone and a plurality of second bases capable of base-pairing with a target nucleic acid;

wherein an antisense analog consisting of a first analog coupled to a second analog is capable of binding to a target nucleic acid and serving as an endonuclease substrate.

45. The library of claim 44, wherein said first analog alone, and said second analog alone, exhibit substantially less biological activity than when combined.

46. The library of claim 44, wherein said first binding domain comprises about 4 to about 12 bases, and said second binding domain comprises about 6 to about 16 bases.

47. The library of claim 46, wherein said first binding domain comprises about 6 to about 8 bases, and said second binding domain comprises about 6 to about 8 bases.

48. The library of claim 47, wherein up to 50% of the second binding domain bases are selected from the group consisting of degenerate bases and universal bases.

49. The library of claim 47, wherein up to 50% of the first binding domain bases are selected from the group consisting of degenerate bases and universal bases.

50. A library of antisense precursor compounds, comprising a plurality of compounds of formula 2 and a plurality of compounds of formula 3:

R_1-L_1-X (Formula 2)

Y-L_2-R_2 (Formula 3)

wherein

R_1 and R_2 are each independently an oligonucleotide or an oligonucleotide analog, capable of binding to mRNA;

L_1 and L_2 are each independently a linking moiety or a bond;

X and Y are each independently a coupling moiety; and

wherein said compounds of formula 2 and formula 3 can be coupled to form a compound capable of recruiting or activating a nuclease when bound to a target polynucleotide.

51. The library of claim 50, wherein said first and second coupling moieties are capable of coupling in the absence of a target polynucleotide.

52. The library of claim 50, wherein X and Y comprise coupling moieties selected from the group consisting of alkyl halides, alkyl sulfonates, activated esters, ketones, aldehydes, amines, hydrazines, sulfhydryls, alcohols, phosphates, thiophosphates, Michael addition receptors, dienophiles, dienes, dipolarophiles, nitriles, alkynes, thiosemicarbazides, isothiocyanates, isocyanates, imidates, and alkenes.

53. The library of claim 50, which comprises at least 1,000 compounds of formula 2 and at least 1,000 compounds of formula 3.

54. The library of claim 53, which comprises at least 10,000 compounds of formula 2 and at least 10,000 compounds of formula 3.

55. The library of claim 50, wherein R_1 and R_2 each independently comprise about 3 to about 24 bases.

56. The library of claim 55, wherein R_1 and R_2 each independently comprise up to about 50% degenerate bases and universal bases.

57. The library of claim 56, wherein R_1 and R_2 each independently comprise about 5 to about 10 bases.

58. The library of claim 57, wherein one of R_1 and R_2 comprises about 8 bases, wherein about 4 of said bases pair specifically with bases in a target polynucleotide.

59. The library of claim 58, wherein said library comprises from about 1,000 to about 8,000 compounds of one of Formula 2 and Formula 3, and from about 100 to about 1,000 compounds of the other formula.

60. A library of ribozyme precursor compounds, comprising a plurality of compounds of formula 4 and a plurality of compounds of formula 5:

$$\text{GG-}R_1\text{-CUGAUGA-}L_1\text{-X} \quad \text{(Formula 4)}$$

$$\text{Y-}L_2\text{-GAA-}R_2 \quad \text{(Formula 5)}$$

wherein

R_1 and R_2 are each independently an oligonucleotide or an oligonucleotide analog, capable of binding to RNA;

L_1 and L_2 are each independently a linking moiety or a bond;

X and Y are each independently a coupling moiety; and

wherein said compounds of formula 4 and formula 5 can be coupled to form a ribozyme.

61. A method for determining an optimal antisense site for a given mRNA, comprising:
selecting a plurality of first oligonucleotide analogs, said first analogs comprising a first coupling moiety and a first binding domain which is complementary to said mRNA;
selecting a second oligonucleotide analog for each first oligonucleotide analog, said second analog comprising a second coupling moiety capable of binding said first coupling moiety, and a second binding domain which is complementary to said RNA at a position proximal to the site to which said first binding domain is complementary;
coupling said first coupling moieties and said second moieties to provide a plurality of antisense probes;
contacting said mRNA with said antisense probes in the presence of an Rnase to form a cleavage product; and
determining which antisense probe corresponds to said cleavage product.

62. The method of claim 61, wherein said first oligonucleotide analogs and said second oligonucleotide analogs are selected from a pre-existing library of oligonucleotide analogs.

63. The method of claim 61, wherein said second bonding domains comprise about 1 to about 4 oligonucleotide bases selected from the group consisting of degenerate bases and universal bases.

64. The method of claim 61, wherein said first bonding domains comprise about 1 to about 4 oligonucleotide bases selected from the group consisting of degenerate bases and universal bases.

65. A method for determining an optimal ribozyme cleavage site for a given target RNA, comprising:

selecting a plurality of first oligonucleotide analogs, said first analogs comprising a first coupling moiety and a first binding domain which is complementary to said target RNA;

selecting a second oligonucleotide analog for each first oligonucleotide analog, said second analog comprising a second coupling moiety capable of binding said first coupling moiety, and a second binding domain which is complementary to said RNA at a position proximal to the site to which said first binding domain is complementary;

coupling said first coupling moieties and said second moieties to provide a plurality of ribozymes;

contacting said target RNA with said ribozymes to form a cleavage product; and

determining which ribozyme corresponds to said cleavage product.

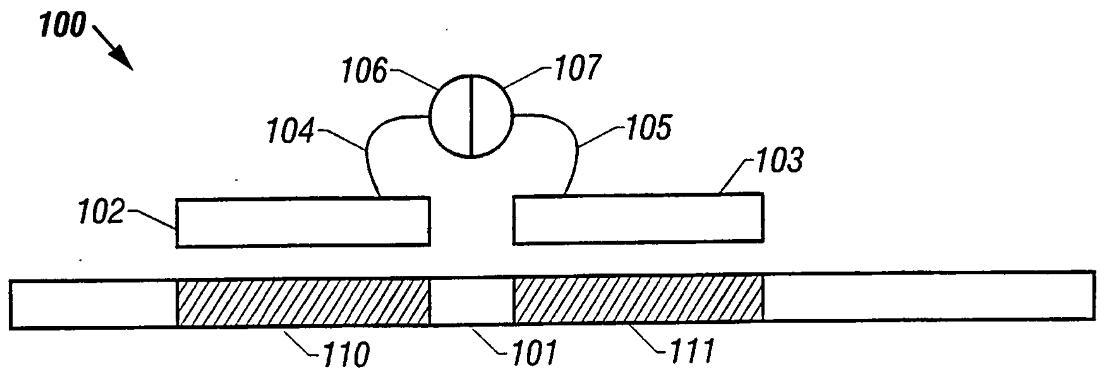

100
106
107
104
105
103
102
110
101
111

FIG. 1

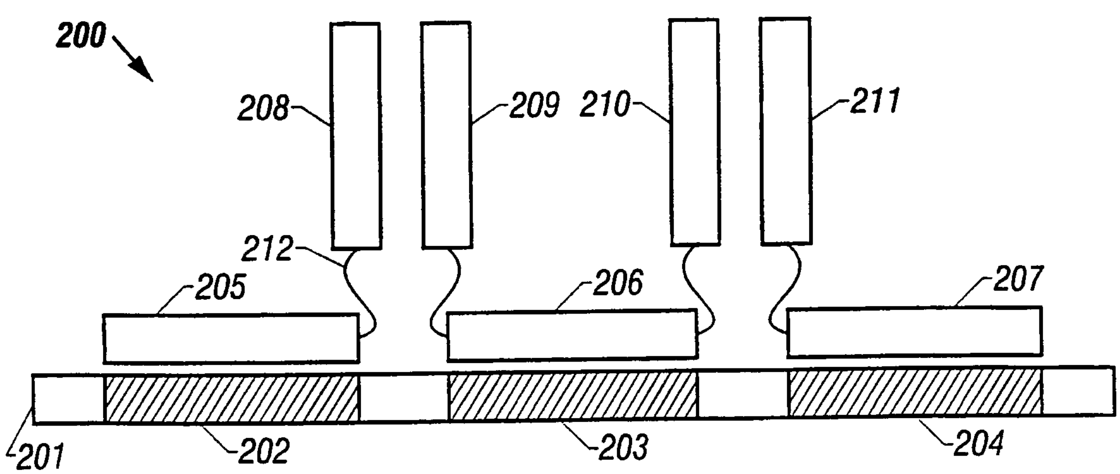

200
208
209
210
211
212
205
206
207
201
202
203
204

FIG. 2

```
                        5' CG 3'
                           GC
                           AU
                           CG
                           CG
                           AU
                  1001     AU   1000
                 Anchor    CG  Cleaver
                           UA
                           CG
                           AU
                           UA
                           GC
           RNA binding     AU   RNA binding/RNase H
              Domain       GC        Substrate
   Target        |         99           |
     |       3'-gcuuaagcggguggTACGACGACGAC-5'
5'-gggcuucgaaucgcgaauucgcccaccaugcugcugcugcugcugcugcugcu...3'
```

FIG. 3

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/20361

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12Q 1/68; C12P 19/34; C12N 15/11, 15/85; C07H 21/04
US CL :435/6, 91.3, 325, 366, 371, 375; 536/23.1, 24.3, 24,33, 24.5
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 91.3, 325, 366, 371, 375; 536/23.1, 24.3, 24,33, 24.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DIALOG, MEDLINE, BIOSIS, DERWENT WPI, APS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X - Y	REYNOLDS, M.A. et al. Antisense Oligonucleotides Containing an Internal, Non-nucleotide-based Linker Promote Site-specific Cleavage of RNA. Nucleic Acids Research. 15 February 1996, Vol. 24, No. 4, pages 760-765, especially pages 760, 763, Figure 1.	1-7, 21-24, 36-38, 42 ------------- 10-20, 26-35, 39-41, 44-59, 61-64
X,P ---- Y,P	US 5,728,818 A (WINCOTT et al) 17 March 1998, col. 2, lines 25-36, col. 5, line 36-col. 6, line 15 and Figures 7-13.	1-5, 9-12, 21-23, 25, 32, 36-40, 42, 43 --------- 13-20, 26-31, 33-35, 41, 60, 65

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date		
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search 17 JANUARY 1999	Date of mailing of the international search report 02 FEB 1999
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer MARK SHIBUYA Lawrence for
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/20361

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X - Y	HENDRY, P. et al. Using Linkers to Investigate the Spatial Separation of the Conserved Nucleotides A9 and G12 in the Hammerhead Ribozyme. Biochimica et Biophysical Acta, Gene Structure and Expression. 18 October 1994, Vol. 1219, No. 2, pages 405-412, especially pages 405-407, figure 1, and 410.	1-5, 9-16, 21-23, 25, 32-40, 42, 43 ----------- 17-20, 26-31, 41, 44-60, 65
X,P ---- Y,P	US 5,686,242 A (BRUICE et al.) 11 November 1997, col. 2, lines 17-65, col. 5, line 39-col. 6, line 11, col. 8, lines 19-51, col. 16, lines 11-60.	1-7, 21-24, 36-38, 44-47, 50, 51, 53-55, 58, 59, 61, 62 ---------- 10-20, 26-35, 39-41, 48, 49, 52, 56, 63, 64
X	KRUPP, G. Antisense Oligoribonucleotides and RNase P. A Great Potential. Biochimie. May 1993, Vol. 75, No. 1/2, pages 135-139, especially page 138, Figure 2.	6, 8
X	LIEBER, A. et al. Selection of Efficient Cleavage Sites in Target RNAs by Using a Ribozyme Expression Library. Molecular and Cellular Biology. January 1995, Vol. 15, No. 1, pages 540-551, especially pages 540-541, 543, Figure 1, 545, Figure 5, 546, 550.	60, 65

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/20361

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/20361

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claims 1-22, and claims 36-41, drawn to a composition comprising a first oligonucleotide analog and second oligonucleotide analog, and a method for cleaving a target RNA molecule using Rnase.
Group II, claims 23-35, drawn to a compound of formula 1: R_1-L_1-X-A-Y-L_2-R_2.
Group III, claims 42-43, drawn to a method of cleaving a target polynucleotide.
Group IV, claims 44-49, drawn to an antisense library.
Group V, claims 50-59, drawn to a library of antisense precursor compounds, comprising plurality of compounds of formula 2, and a plurality of compounds of formula 3.
Group VI, claim 60, drawn to a library of ribozyme precursor compounds, comprising plurality of compounds of formula 4, and a plurality of compounds of formula 5.
Group VII, claims 61-64, drawn to a method of determining an optimal antisense site for a given mRNA.
Group VIII, claim 65, drawn to a method of determining an an optimal ribozyme cleavage site for a given target RNA.

The inventions listed as Groups I to VIII do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: the special technical feature of Group I invention is a composition comprising two oligonucleotides which bind to target polynucleotide and in the absence of target oligonucleotide capable of coupling and a method for cleaving a target RNA using Rnase, whereas the special technical feature of Group II is a compound of formula I claimed which is capable of activating a nuclease, the special technical feature of Group III is a method for cleaving a target polynucleotides is claimed, the special technical feature of Group IV is an antisense library claimed, the special technical feature of group V is a library of antisense precursor compounds of formula 2 and compounds of formula 3, the special technical feature of Group VI is a library of ribozyme precursor compounds of formula 4 and compound of formula 5, the special technical feature of Group VII is a method for determining an optimal antisense site for a given mRNA, the special technical feature of Group VIII is a method for determining an optimal ribozyme cleavage site for target RNA. Since the special technical feature of group I is not present in Groups II-VIII inventions, and the special technical features of Groups II-VIII are not present on Group I, unity of invention is lacking.